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Small-Scale Studies of Microbiological Oxidation of Hydrocarbons to Long-Chain Fatty Acids

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Summary. Several strains of *Pseudomonas, Nocardia* and incompletely identified soil isolates have been grown in a mineral salts plus hydrocarbon medium, and the fatty acids produced by the organisms have been isolated, identified and estimated. The results of an estimation of the percentage conversion to these acids under varying experimental conditions is discussed in relation to the metabolic systems involved. Some indication has been obtained that the hydrocarbon breakdown pathway by these organisms is that of ω oxidation followed by β oxidation. Preliminary experiments carried out with one strain of organism, *Pseudomonas aeruginosa* 5940, indicate that there may be some difference in the utilization of odd- and even-chain hydrocarbons by this organism. An improvement of 3·8-fold was obtained by using nitrate instead of ammonium nitrogen; 13-fold by using continuous instead of batch operation; and 8-fold by the use of liquid instead of solid paraffins, giving a total improvement of yield of 400-fold.

Introduction

The ability of micro-organisms to utilize hydrocarbons as a sole carbon source has been recognized for many years. The interest in this field has chiefly been associated with the breakdown of petroleum and petroleum products, and with extension of the knowledge on the spectrum of carbon sources used by various species of organisms. Later workers have studied the pathway of hydrocarbon oxidation.

This particular work was initiated to find out if a useful oxidation of hydrocarbons, such as paraffin wax and liquid paraffin, could be carried out to yield a reasonable amount of long-chain saturated fatty acids, such as stearic and palmitic acids; this work relates to the long-chain fatty acids only, and takes no account of any short-chain compounds, or of a general breakdown of hydrocarbons such as is encountered in the treatment of industrial wastes carrying these substrates. Reference to a review by Zobell (1946) and the book on *Petroleum Microbiology* by Beerstecher (1954)

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indicated that organisms belonging to the genera *Pseudomonas* and *Nocardia* might prove useful for the task. A preliminary report of a demonstration of this work has already been published (Linday, 1961).

Previous workers have detected fatty acid production from hydrocarbons (Bushnell and Haas, 1940; Haas, Yantzi and Bushnell, 1941; Johnson, Goodale and Turkevitch, 1942) using fall in pH as the criterion of acid formation. Strawinski (1943), and Strawinski and Stone (1943) have isolated *ortho*-salicylic acid as the oxidation product of cultures of *Pseudomonas aeruginosa* growing on naphthalene. Other studies on microbial action on aromatic compounds have been made by Arnaudi, Canonica and Treccani (1955) and Colla, Fiecchi and Treccani (1959).

The pathway of microbial breakdown of hydrocarbons has been studied by several workers. Webley and his co-workers have studied hydrocarbon oxidation by *Nocardia opaca*, using phenolic groupings as markers for the course of reaction (Webley, Duff and Farmer, 1955; Webley, Duff and Farmer, 1959), and have found it to follow the suggested pathway of Breusch (1948) of end chain or ω oxidation to give fatty acids, followed by β oxidation, resulting in the systematic shortening of the fatty acid chains by C_2 units. Stewart, Kallio, Stevenson, Jones and Schissler (1959) and Stewart and Kallio (1959), working with an unidentified Gram-negative coccus, have suggested another pathway for the conversion of alkanes, namely to esters (for example, if *n*-hexadecane is fed as sole carbon source to this organism, they isolate cetyl palmitate as the main product of microbial oxidation).

In this work, hydrocarbon oxidation by various pure cultures of bacteria, and unidentified soil isolates, has been studied, the actual long-chain fatty acids resulting from the reaction have been identified and estimated, and their variation in the percentage composition of the product with time, nitrogen supply, and other environmental conditions, has been investigated.

Materials and Methods

General

Cultures of *Pseudomonas* were obtained from the National Collection of Type Cultures, and from the National Collection of Industrial Bacteria. A culture of *Nocardia opaca* was obtained

from the Canadian Department of Agriculture, Ottawa, and one of *Nocardia petrofila* from Dr. Peter Hirsch (Hirsch, 1957). Unidentified hydrocarbon-oxidizing bacteria were isolated from soil samples by elective culture techniques in a medium of mineral salts and hydrocarbon.

The culture medium used consisted of a mineral salts solution with an inorganic nitrogen source, and hydrocarbon as sole carbon source. The mineral salts solutions used for all cultures were either the mixture given by Webley (1954) in which the nitrogen supply was $NaNO_3$, or, in experiments where the type of nitrogen supply was altered, $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ used in such concentration that the nitrogen present was equal in amount to that in the $NaNO_3$ medium; and other ions present were also adjusted to this medium. Distilled water was used in this medium. The pH for all culture media, unless stated otherwise, was 6·9–7·2. Hydrocarbon sources were liquid paraffin BP (Hopkin and Williams Ltd.), paraffin wax (Shell Chemical Co. Ltd.), *n*-docosane (C_{22}) (L. Lights Ltd.), *n*-octadecane (C_{18}) (British Drug Houses Ltd.) and some pure hydrocarbons of known structure kindly supplied by British Petroleum Co. Ltd. Sodium succinate used in some experiments was of technical grade, otherwise all other chemicals used were of Analar grade.

Stock cultures were maintained on nutrient agar slopes, and stored in the refrigerator. Seed cultures were maintained in mineral salts–liquid paraffin medium; after initial inoculation into the liquid paraffin medium, it was not necessary to re-seed from the stock cultures, but these were retained for checking purposes.

Seed cultures and small-scale experimental cultures were grown in cotton-wool-plugged conical flasks; seed cultures in an incubator set at 25°C with the flasks shaken night and morning; and experimental cultures in a constant-temperature shaking tank with the thermostat set at 25°C. Pilot-plant-scale work was done on a 2–3 l. batch/continuous fermentor of the type described by the Porton group (Elsworth, Meakin, Pirt and Capell, 1956; Elsworth, Capell and Telling, 1958). In large-scale experiments the temperature was controlled at 25°C, and aeration was achieved by stirring and passing air so that the level obtained was equivalent to a sulphite oxidation of 170 mmoles/l./h (Elsworth, Williams and Harris-Smith, 1957). Centrifuging was carried out at 10°C for

30 min at $2,000 \times g$. Growth determinations were made, where possible, by a turbidity method; when this was not possible, because of the nature of some of the media, a dry-weight determination was made. It was not found advisable to add a surface-active agent to increase emulsification of the substrates, as this produced excessive foaming. However, natural emulsification occurred after 1 day's growth, presumably due to the fatty acids produced during metabolism.

Extraction Techniques

Extraction of the fatty acids from the medium after removal of the cells was as follows.

The medium was made strongly acid with concentrated HCl and then extracted with ether or chloroform, and the ether layer retained. The ether solution was then treated with aqueous NaOH, and the fatty acids passed into the aqueous phase leaving unused hydrocarbons in the ether layer. The alkaline solution was reacidified with HCl and extracted with ether or chloroform; at this stage the free fatty acids pass into the solvent layer, which was separated and dried with anhydrous Na_2SO_4. Then the ether was removed by distillation and became known as the fatty acid fraction sample. In some experiments a full alkaline hydrolysis was carried out on the initial solvent extract, but this was not found to give a very much better yield of fatty acids from unused hydrocarbons. The conditions for alkaline hydrolysis were: reflux the fatty material in ethyl alcohol and KOH for at least 4 h, remove the alcohol, and add water to the residual material and extract it with ether or chloroform. The non-saponifiables pass into the solvent layer and can be removed. The aqueous layer is then acidified and extracted with ether, and at this stage the fatty acids pass into the ether layer, which is dried, and concentrated.

At the end of a run, either batch or continuous, the fermentor was steamed out so as to collect all hydrocarbons and other substances from the walls of the fermentor, and then this steam condensate was added to the liquor for extraction.

Analytical Techniques

Analysis of free fatty acids was carried out on reversed-phase paper chromatograms running in a developing solvent of 80 per

cent acetone, 20 per cent water, with an alkaline, alcoholic Nile Blue sulphate spray technique for showing up the spots (Chayen and Linday, 1960).

The formation of the carbonyl grouping could be followed on an infrared spectrometer, using Nujol (liquid paraffin) mull preparations of the extract. In some long-term experiments with liquid paraffin as substrate, the course of the metabolism was followed by infrared analytical techniques. A sample of 50 ml was removed each day from the fermentor, centrifuged to remove the

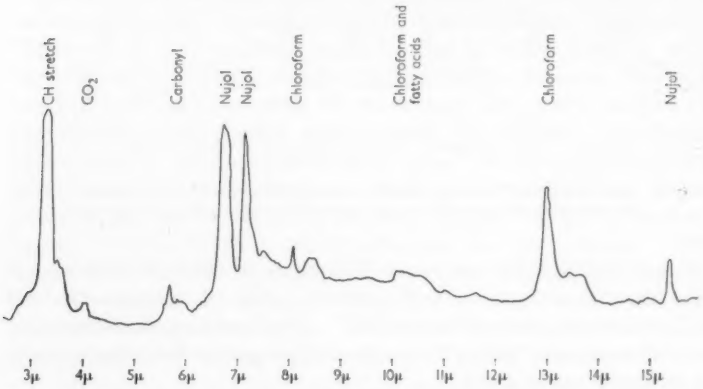

Fig. 1. Infrared spectrometer trace of a chloroform extract of the acidified growth medium. This trace shows the presence of the carbonyl grouping

cells, acidified with concentrated HCl, and then extracted with chloroform. The chloroform extract was dried over anhydrous Na_2SO_4, and then concentrated until all chloroform was removed. The sample was then run on an infrared spectrometer and the development of the carbonyl grouping peak followed. When this peak showed no further increase in proportion to the Nujol peaks, the experiment was stopped, and the whole reactor contents extracted and prepared for a final analysis of fatty-acid content by vapour-phase chromatography. The type of trace obtained by infrared analysis is illustrated in Fig. 1.

Methyl esters of fatty acids were identified and estimated on a Pye argon-vapour-phase chromatograph in which a 4-ft 15 per cent Apiezon L grease–Celite column was used at 190°C, at the $\times 10$

sensitivity and 1250-V detector voltage setting, and with an argon flow rate of 20 ml/min and a chart speed of 15 in./h. A typical trace of the methyl esters and wax are shown in Fig. 2. It is necessary to methylate the long-chain fatty acids before analysing them by vapour-phase chromatography, as the boiling points

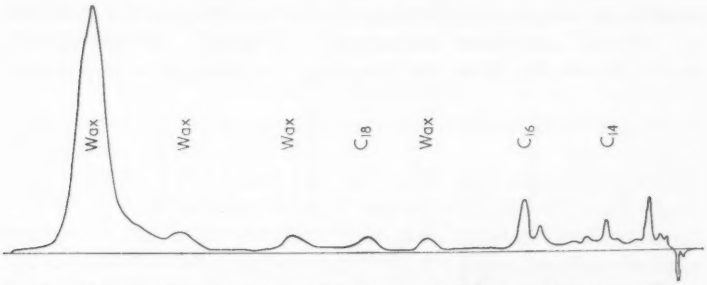

Fig. 2. Vapour-phase chromatograph of methylated fatty-acid extract. Trace shows peaks of wax and methyl esters of C_{18}, C_{16} and C_{14} fatty acids

of the methyl esters are lower than those of the free acids and so the analysis of these, up to C_{18}, can be achieved in a time of about 1 h at a temperature of 190–200°C. It is hard to find a reasonably stable stationary phase for a column to separate fatty acids above a temperature of 200°C.

Methylation of fatty acids was carried out by refluxing them with methanol and concentrated H_2SO_4 for at least 3 h. The methanol was then distilled off, the residue diluted with water, and ether extracted. The ether layer was dried with anhydrous Na_2SO_4 and concentrated. The methylated fatty acids were passed through a silica-gel column to remove any traces of hydrocarbons; this technique is the one described by Howard (1957).

Note on Types of Hydrocarbon Structures Used

Liquid paraffin and paraffin wax. Mixed hydrocarbons. Liquid paraffin, s.g. 0·89.

n-Octadecane, $C_{18}H_{38}$. Straight-chain hydrocarbon.

n-Docosane, $C_{22}H_{46}$. Straight-chain hydrocarbon.

n-Tetracosane, $C_{24}H_{50}$. Straight-chain hydrocarbon.

2-Methylheptadecane, $C_{18}H_{38}$. C_{17} chain with a methyl group on the second carbon.

2,21-Dimethyldocosane, $C_{24}H_{50}$. Two methyl groups at points indicated on the straight chain.

3,3,6,6,9,9,12,12-Octamethyltetradecane, $C_{22}H_{46}$. Eight methyl groups at points indicated on the straight chain.

n-Pentadecylcyclohexane, $C_{21}H_{41}$. Alicyclic (ring) hydrocarbon.

The rate of hydrocarbon breakdown by oxygen uptake was measured on a normal Warburg apparatus. The cells used in these experiments were grown under the conditions stated, then harvested by centrifuging, washed twice in buffer solution with centrifuging, and finally resuspended in buffer solution. The cells were then shaken in buffer for 6 h before they were used in an experiment. The buffer used was M/30 Sorensen phosphate buffer pH 7·2. In each experiment 2 ml of cells in buffer were used in the main well of the flask, 0·2 ml of 1 per cent KOH and filter paper in the centre well, and the hydrocarbon, or water (control flasks), in the side arm. Air was the gas used in all flasks. The temperature for the experiments was set at 25°C, and the shaking rate 120 strokes per minute. Each hydrocarbon test or control was made in duplicate flasks. The rate of hydrocarbon oxidation is expressed as μl. O_2 uptake/h/mg dry weight of cells.

Results

Preliminary Experiments with Pseudomonas *sp.*, Nocardia *sp., and Soil Isolates*

Preliminary experiments were carried out to select the most suitable strain of organism for large-scale work. The strains were grown in mineral salts medium (Webley, 1954) with liquid paraffin as hydrocarbon source. Each experimental set of flasks consisted of (1) a mineral-salts medium, without hydrocarbon, inoculated with the organism, (2) a mineral-salts medium, with hydrocarbon, inoculated with the organism, and (3) a mineral-salts medium, with hydrocarbon, uninoculated. The flasks were shaken, and the

change in pH followed on samples with a pH meter. The results are presented in Table I.

Table I. Change in pH of hydrocarbon–mineral salts media during the growth of various species[a]

Strain	Medium[b]	Growth	pH initial, all flasks	pH final[c] 1	2	3	Time, days
Nocardia opaca	M.S.+L.P.	+	7·2	7·2	7·4	7·2	12
Nocardia petrofila	M.S.+L.P.	−	7·2				12
Pseudomonas NCTC 950	M.S.+L.P.	+	7·2	7·2	7·6	7·2	12
Pseudomonas NCIB 8250	M.S.+L.P.	+	7·2	7·2	7·6	7·2	12
Pseudomonas NCIB 6576	M.S.+L.P.	+	7·2	7·2	6·8	7·2	12
Pseudomonas NCIB 8768	M.S.+L.P.	+	7·2	7·2	6·8	7·2	12
Pseudomonas NCIB 8295	M.S.+L.P.	+	7·2	7·2	6·8	7·2	12
Pseudomonas NCIB 8929	M.S.+ C_{18} wax	+	6·8	6·8	6·6	6·8	12
Pseudomonas NCIB 8027	M.S.+L.P.	+	7·2	7·2	7·2	7·2	11
Pseudomonas NCTC 5940	M.S.+P.W. or L.P.	+	7·2	7·2	5·0	7·2	11
Sarcina lutea NCTC 11	M.S.+L.P.	+	7·2	7·2	6·0	7·2	11

[a] Incubation temperature, 25°C.
[b] M.S. = Mineral salts; L.P. = liquid paraffin; P.W. = paraffin wax; C_{18} = *n*-octadecane.
[c] 1, Mineral salts inoculated; 2, mineral salts + hydrocarbon inoculated; 3, mineral salts + hydrocarbon, uninoculated.

The strains showing the largest fall in pH were used in further studies, particularly *Pseudomonas aeruginosa* 5940.

Table II shows that with *Pseudomonas aeruginosa* 5940, a starting

Table II. Change in pH produced by *P. aeruginosa* 5940 in hydrocarbon–mineral salts medium at various initial pH values[a]

Strain	Medium[b]	Growth	pH initial, all flasks	pH final 1	2	3	Time, days
NCTC 5940	M.S.+P.W.	+	6·2	6·2	4·4	6·2	12
Pseudomonas aeruginosa		+	6·8	6·8	4·4	6·8	
		+	7·2	7·2	5·0	7·2	
		+	7·8	7·8	6·15	7·8	
		+	8·2	8·2	6·2	8·2	

[a] Incubation temperature, 25°C.
[b] M.S. = Mineral salts; P.W. = paraffin wax.

pH of 6·8–7·0 produces the best result in a similar experimental set-up to that given above. Tests on the effect of nitrogen source on this organism indicated a better growth rate and level

with nitrogen sources other than nitrate; but later large-scale experiments, although bearing out this observation, showed that fatty-acid production was higher in a nitrate medium than in an ammonium medium.

Large-scale Experiments with Pseudomonas *sp. and Soil Isolates*

Large-scale experiments were carried out on both batch and continuous processes. The results are presented in Table III, and show the strain of organism used, the time of growth, and the conditions of the process. In two-stage experiments, the organisms were grown in the mineral-salts solution with sodium succinate as carbon source. The cells were harvested by centrifuging, washed with buffer solution (M/30 Sorensen phosphate buffer pH 7·2) and then returned to the fermentor, and the experiment continued with a medium of mineral salts plus hydrocarbon.

A check was kept during the experiments to see that hydrocarbon, nitrogen and phosphorus sources did not reach too low a value. The hydrocarbon content could be estimated during vapour-phase-chromatography analysis of the samples, as the wax peaks showed on the trace obtained. The nitrogen content of the medium was estimated by the method of Conway (1947), and the phosphorus content by Allen's (1940) modification of the method of Fiske and Subarrow. It also appeared probable that oxygen itself (or rather the transfer of oxygen to the reacting enzyme sites) might be a limiting factor in this system. A minor attempt to test this was made in which duplicate fermentors were run under identical conditions, except that the gas inflow to one was 1 l. of air per minute, and to the other was 0·8 l. air + 0·2 l. O_2 per minute. There was no appreciable difference in the product results of the two types of fermentation.

Analysis of the products of the growth on the hydrocarbon was made by the methods described. In experiments where *n*-docosane (C_{22} wax) was the substrate, then the products of metabolism in the fatty acid fraction were the even-chain fatty acids. The products when paraffin wax and liquid paraffin were used as substrate were more complex, and here the analysis was conducted in such a manner as to estimate only the proportion of long-chain fatty acids resulting from the organism's metabolism, as the purpose of the research was to obtain a reasonable yield of these

Table III. Selection of results showing % of C_{18}, C_{16} and C_{14} methyl esters in the fatty-acid fraction of the solvent extract of hydrocarbon–mineral salts medium in which various organisms have been grown under different conditions in a fermentor[a]

Expt. no.	System	Stages	N_2 Source 1	N_2 Source 2	Hydrocarbon	Hydrocarbon Amount fed, g	Hydrocarbon Amount recovered, g	Organism	Days	Fatty-acid fraction, g	Methyl ester %C_{18}	Methyl ester %C_{16}	Methyl ester %C_{14}	% Conversion
10	Batch	2	NO_3	NO_3	C_{22} Wax	14	11	5940	1	0·1	0·34	0·5	0·22	0·035
									2	0·1	0·77	0·92	1·23	0·097
									3	0·05	0·47	1·03	0·56	0·034
13	Batch	2	NO_3	NO_3	C_{22} Wax	10	5	5940	1	0·05	0·6	0·62	0·02	0·012
									2	0·025	1·76	2·62	0·34	0·024
									3	0·05	2·74	4·0	1·73	0·085
									4	0·1	0·18	0·82	0·45	0·029
14	Batch	2	NO_3	NH_4	C_{22} Wax	15	4·9	5940	1	0·2	—	—	2·175	0·043
									2	0·1	2·0	3·2	2·0	0·071
									3	0·15	—	7·7	5·4	0·19
									4	0·15	1·0	4·8	0·245	0·08
15	Batch	2	NH_4	NH_4	C_{22} Wax	15	9·4		1	0·05	—	—	1·02	0·009
									2	0·02	3·7	6·5	0·98	0·036
									3	0·06	0·95	0·71	0·02	0·018
									4	0·07	—	—	—	—
24	Batch	1	NO_3		Liq. Par.	44·5	30·1	'Old Soil'	9	0·5	2·26	5·75	7·05	0·52
25	Batch	1	NO_3		Liq. Par.	44·5	34·4	'Professor'	5	1·1	—	6·9	5·8	1·15
32	Batch	1	NO_3		C_{22} Wax	7·8	4·0	'Sand Oil'	2	1·2	0·4	0·6	0·08	0·314
40	Cont.		NO_3		Liq. Par.	195·0	155·0	5940		4·5	23	1·5	22·7	5·31
41	Cont.		NO_3		Liq. Par.	193·0	157·0	5940		2·0	—	16·2	59·0	4·17
44	Cont.		NO_3		Par. Wax	50·0	37·0	5940		0·4	5·1	7·7	6·6	0·59

[a] 5940 = *P. aeruginosa* 5940; Liq. Par. = liquid paraffin; Par. Wax. = paraffin wax; C_{22} Wax = *n*-docosane; Cont. = semi-continuous system.

substances from such substrates. Thus, in Table III the proportion of C_{18}, C_{16} and C_{14} fatty acids in the fatty-acid fraction is the figure given.

Comments on the Soil Isolates

These isolates were obtained from various soil samples by elective culture techniques using a mineral salts–liquid paraffin growth medium, and then the isolates were separated into pure culture by plating methods, and identification procedure followed to a limited extent.

Old Soil. Gram-negative, motile, bacillus, possibly a species of *Pseudomonas.*

Professor. Gram-positive bacillus, possibly a species of *Bacillus.*

Sand-Oil. Gram-positive bacillus, possibly a species of *Arthrobacter.*

Experiments Using Pure Hydrocarbons of Known Structure

The organism used in these experiments was *Pseudomonas aeruginosa* 5940. The hydrocarbons used were *n*-tetracosane, 2-methylheptadecane, 2,21-dimethyldocosane, 3,3,6,6,9,9,12,12-octamethyltetradecane and *n*-pentadecylcyclohexane and *n*-octadecane, and as only small amounts of some compounds were available the experiments had to be somewhat limited. The experiments were of two types: growth experiments in mineral salts–hydrocarbon medium on the plan used in other sections of the work, and Warburg experiments in which the rate of oxidation of the hydrocarbon by the organism was measured. The conditions used in the Warburg experiments are stated in the section on methods.

The results of the growth experiments are presented in Table IV and show that straight-chain hydrocarbons of both odd (this had a C_{17} straight chain with a single methyl group side chain) and even carbon number are utilized by the organism, and also that branched-chain hydrocarbons (2,21-dimethyldocosane and 3,3,6,6,-9,9,12,12-octamethyltetradecane) can also be metabolized. The organism did not, however, grow on the ring compound (*n*-pentadecylcyclohexane) under the experimental conditions, but after prolonged incubation in this substance some growth did occur.

* 5940 = *P. aeruginosa* 5940; Liq. Par. = liquid paraffin; Par. Wax. = paraffin wax; C_{24} Wax = *n*-docosane; Cont. = semi-continuous system.

Table IV. Growth and pH changes in a mineral-salts media with various types of hydrocarbon as sole carbon source[a]

Hydrocarbon	Growth[b]	Time, days	Initial pH	Final pH[c] 1	2	3
n-Tetracosane	+	4	6·9	6·95	8·2	6·95
2-Methylheptadecane	+	3	6·8	6·85	7·0	6·8
2,21-Dimethyldocosane	+	4	6·9	6·95	8·1	6·95
3,3,6,6,9,9,12,12-Octamethyltetradecane	(+ −)	3	6·8	6·85	7·0	6·8
n-Pentadecylcyclohexane	(+ −)	4	6·9	6·95	7·2	6·95

[a] *P. aeruginosa* 5940; temperature, 25°C.
[b] + = Growth; (+ −) = little or no growth.
[c] 1, Mineral salts inoculated; 2, mineral salts + hydrocarbon inoculated; 3, mineral salts + hydrocarbon uninoculated.

The even straight- and branched-chain hydrocarbons, *n*-tetracosane and 3,3,6,6,9,9,12,12-octamethyltetradecane are oxidized directly by starved suspensions of this organism which has been grown in nutrient broth. The ring compound however is not oxidized. Table V shows an interesting comparison between the

Table V. Measurement of O_2 uptake by starved suspensions of *P. aeruginosa* 5940 in phosphate buffer solution, after growth on different substrates and with either odd or even chain hydrocarbons supplied as substrate during the test[a]

Growth substrate[b]	Rate of O_2 uptake μl./h/mg dry wt.		
	Control	C_{17}	C_{20}
Nutrient broth	58	58	61
MS + C_{17}	18	31	24
MS + C_{22}	18	25	23

[a] Temperature, 25°C.
[b] M.S. = Mineral salts; C_{17} = 2-methylheptadecane; C_{22} = *n* − docosane.

behaviour of this organism on the odd- and even-chain-length hydrocarbons. The organism, when grown in nutrient broth and starved in buffer, will directly oxidize the even-chain hydrocarbon (*n*-docosane) but not the odd-chain one (2-methylheptadecane). However, the organism will grow on both the odd- and even-chain hydrocarbons, and then starved suspensions of the cells from either type of medium will oxidize both odd- and even-chain hydrocarbons.

The results given in this section are only preliminary observations, and further investigations are in progress, particularly in

relation to the phenomenon observed with the odd-chain hydrocarbon.

Discussion

The results obtained with various species of organism growing on the hydrocarbons liquid paraffin and paraffin wax indicate that long-chain fatty acids are produced as a result of the organism's metabolism. The results of the experiments carried out with the organism on the C_{22} wax would seem to lend support to the theory that the path of hydrocarbon oxidation is one of ω oxidation followed by β oxidation (Breusch, 1948), since the fatty acids isolated and identified from the growth medium with this hydrocarbon included C_{18}, C_{16} and C_{14}; but much more detailed work with tracer techniques, cell-free enzyme preparations, and isolation of intermediate compounds is required before any real evidence in this direction could be claimed. Some additional support for this theory also came from experiments in which stearic or palmitic acid were used as the sole carbon source in a mineral-salts medium. The products of reaction isolated from this medium were found to be the acids, in homologous series, with 2 carbon atoms less at each stage. This attack on the acids themselves probably also accounts for the low and varied yields of the fatty acids.

The organism *Pseudomonas aeruginosa* 5940 appears to be able to utilize a wide range of hydrocarbon structures as sole carbon source. The preliminary experiments carried out with this organism on odd- and even-chain-length hydrocarbons seem to indicate that there may be some difference in the utilization of these two types of structure.

In dealing with continuous systems, it was very much easier from the point of view of handling the feed systems to use liquid paraffin as the hydrocarbon source than it was to use one of the waxes. However, analysis of products from wax systems was far easier to carry out accurately as peak separation was clearly defined. The continuous system was of a semi-continuous nature and was operated on the following basis. The culture was grown for 3 days batchwise, then the feed systems were brought into operation and the flow set to 2 l. per 24 h, at which rate the condition was maintained constant. The pH was controlled through-

out the experiment to 6·8. The overflow from the system was fed into collecting vessels where the pH was immediately dropped to 1, and then the liquor was treated as described in the Methods section.

In Table III there is a column of figures labelled '% conversion'. These figures were obtained by the following type of calculation, taking as an example Experiment No. 25 in Table III. Here the total C_{18}, C_{16} and C_{14} methyl esters of fatty acids is 1·08 per cent of the fatty-acid fraction of the extract from the medium, which itself weighed 1·2 g. The loss of hydrocarbon during the experiment was 3·8 g. Then the percentage conversion to the methyl esters on the basis of hydrocarbon loss was:

$$\frac{1{\cdot}08 \times 1.2}{3{\cdot}8} = 0{\cdot}314 \text{ per cent}$$

If comparisons are made between the percentage conversions obtained by the different experimental conditions, the following conclusions can be drawn from all the results obtained.

In batch processes using *Pseudomonas aeruginosa* 5940 and wax as a hydrocarbon source, the average conversion to the methyl esters was 0·012 per cent when ammonium nitrogen was used as nitrogen source, as compared with a figure of 0.045 per cent when nitrate nitrogen was used as nitrogen source. When *Pseudomonas aeruginosa* was grown batchwise with a nitrate nitrogen source and a wax hydrocarbon source, then the average conversion to the methyl esters was 0.045 per cent, as compared with the figure of 0·59 per cent when the same system was continuous. It is very difficult to draw valid comparisons for continuous systems using the same organism and nitrogen source but different types of hydrocarbon, namely wax and liquid paraffin, as the physical nature of the sources is so different and mechanical handling becomes a contributory factor in operation of the system. However, for liquid paraffin the average conversion was 4.74 per cent as compared with 0.59 per cent for a wax system.

An explanation of these results is suggested in terms of the attack on the acids themselves mentioned in a previous paragraph of this discussion. This attack probably accounts for the low and varied yields of the acids shown in the results. Growth of the organism is much higher in the presence of ammonium nitrogen, and this would explain why the yield of acids is lower when the

high growth is obtained with this nitrogen source, as compared with the higher yield with the nitrate nitrogen source. The use of the semi-continuous process no doubt aided in preventing some of the attack on the acids, leading to the higher yield obtained in continuous systems compared with batch systems. Some preliminary experiments have been carried out incorporating basic ion-exchange resins in the system in an effort to hold the acids on the resin as they are formed, and thus increase the yield.

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Adsorption Method for Measuring the Specific and Average Areas of Cells

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Summary. This paper presents a method for measuring the specific area (area/mass) of micro-organisms and the average area of cells, based on the adsorption of dyes.

Introduction

The enormous specific area of micro-organisms is, very probably, responsible for many microbiological phenomena (Clifton, 1950; Thimann, 1955). Its correct measurement will certainly play an important rôle in the quantitative interpretation of many facts (Borzani, 1960; Donovick, 1960), especially those regarding the kinetic studies of fermentation processes.

The average area of cells is usually calculated from direct measurement of some linear dimensions, assuming the cells present a well-defined geometrical form and a perfectly smooth surface (Verona, 1956). From the calculated average area and the number of cells that exist in a given mass of micro-organism, the specific area of this micro-organism can be determined.

Obviously, such a method for the calculation of the specific area presents several important disadvantages:

1. It is based on very difficult linear measurements (Knaysi, 1945).
2. A great number of cells must be measured in order to obtain a statistically significant result.
3. The cells never present a defined geometrical form.
4. The cell surface is not perfectly smooth.

The problem is even more complicated when grouped cells or hyphae are considered.

The purpose of this paper is to present an adsorption method for the determination of the specific area of micro-organisms and

for the measurement of the average area of cells, analogous to the methods used for the evaluation of the specific area of adsorbents (Glasstone, 1940a).

Basis and Description of the Method

In previous papers (Borzani and Vairo, 1958; Borzani and Vairo, 1959; Borzani and Vairo, 1960; Borzani and Vairo, 1960a; Vairo, 1961; Vairo and Borzani, 1960) it was verified that the adsorption of certain dyes by micro-organisms follows physico-chemical laws, particularly the adsorption law of Langmuir.

This fact indicates that very probably the tested dyes are adsorbed as a uniform unimolecular layer, since, as it is well known, this is one of the hypotheses for the establishment of the Langmuir isotherm (Glasstone, 1940).

Then, measuring the quantity of dye that saturates, by adsorption, a given mass of cells, and if the area covered by a given mass of dye is known, the specific area of the micro-organism can be determined. From this calculated specific area and the number of cells contained in a certain mass of micro-organism, the average cell surface can easily be evaluated. In fact, if C = concentration of micro-organism; C_i = initial dye concentration; C_f = dye concentration at the equilibrium point (assuming the cell's surface saturation was reached); S = area covered by a unit mass of dye (uniform unimolecular layer); N = number of cells per unit mass of micro-organism; σ = specific area of the micro-organism; and s = average area of the micro-organism cell; the following equations can be established:

$$\sigma = (C_i - C_f)S/C \qquad (1)$$

$$s = \sigma/N \qquad (2)$$

Equations (1) and (2) must be applied when the dye completely saturates the cell surface. This complete saturation can be assured only by extreme experimental conditions, that is, working with very high dye concentrations or with very low cell concentrations. It is well known, however, that experiments of this kind lead to doubtful results, since the values obtained working under such unfavourable conditions are probably affected by relatively high errors. It seems more advisable to realize the measurements

using favourable concentrations of cells and dye, and then extrapolate the results by means of the Langmuir law as follows.

The Langmuir isotherm is:

$$\frac{C_i - C_f}{C} = \frac{aC_f}{1+bC_f}$$

Then equation (1) gives:

$$\frac{\sigma}{S} = \frac{aC_f}{1+bC_f}$$

$$\therefore \frac{S}{\sigma} = \frac{b}{a} + \frac{1}{a}\cdot\frac{1}{C_f}$$

and when $C_f \to \infty$ (dye concentration extremely high), the last equation will give the most probable value of the specific area:

$$\sigma = \frac{a}{b}\cdot S \qquad (3)$$

Equation (2) then permits the calculation of the average area of the cell.

Numerical Examples

The following examples show the application of the proposed method to some particular cases.

Example I. Table I presents the results obtained in adsorption experiments carried out with aqueous solutions of methylene blue and cells of *Saccharomyces cerevisiae* killed by boiling for 10 min; the values given in Table I obey the following Langmuir equation (Vairo, 1961):

$$C/(C_i - C_f) = 0{\cdot}0136 + 0{\cdot}56(1/C_f)$$

where C and C_f are measured in grams of dry matter per litre and in mg per litre respectively.

The value of S for methylene blue is 10^4 cm^2/mg (Paneth and Radu, 1924). Then, equation (3) gives:

$$\sigma_1 = \frac{1}{0{\cdot}0136} \times 10^4 = 74 \times 10^4 \text{ cm}^2/\text{g dry matter}$$

The direct counting of cells leads to the result $N = 3{\cdot}4 \times 10^{10}$

cells per gram of dry matter, and equation (2) then permits the calculation of the average area of a cell of *Saccharomyces cerevisiae*:

$$s_1 = \frac{74 \times 10^4}{3{\cdot}4 \times 10^{10}} = 22 \times 10^{-6}\ \text{cm}^2 = 2.2 \times 10^3\ \mu^2$$

The average area obtained from direct measurement of 160 cells of *Saccharomyces cerevisiae* (assuming the cell is ellipsoidal) was 110 μ^2 (Vairo, 1961), i.e., twenty times smaller than the value obtained by the adsorption method.

Table I. Adsorption of methylene blue by dead cells of *Saccharomyces cerevisiae* (Vairo, 1961)

Cell concentration, C, g dry matter/l.	Dye concentration at the equilibrium point, C_f, mg/l.
0·266	89·0
0·532	77·5
0·798	66·0
1·065	57·9
1·331	48·2
1·597	42·3
1·863	37·3
2·129	33·0

Initial dye concentration = 102·6 mg/l.

Example II. The adsorption of methylene blue by dead cells of *Sarcina lutea* follows the Langmuir law (Borzani and Vairo, 1960):

$$C/(C_i - C_f) = 0{\cdot}0064 + 0{\cdot}10(1/C_f)$$

Then, the specific area of *Sarcina lutea* can be calculated:

$$\sigma_2 = \frac{1}{0{\cdot}0064} \times 10^4 = 156 \times 10^4\ \text{cm}^2/\text{g dry matter}$$

In this case, $N = 1{\cdot}2 \times 10^{13}$ cells per gram of dry matter; and the average area of a cell is given by:

$$s_2 = \frac{156 \times 10^4}{1{\cdot}2 \times 10^{13}} = 13 \times 10^{-8}\ \text{cm}^2 = 13\ \mu^2$$

From direct measurements, the average area of a cell of *Sarcina lutea* was calculated as 4·5 μ^2.

Example III. Adsorption of crystal violet by dead cells of *Saccharomyces cerevisiae* and *Sarcina lutea* (samples different from those used in Examples I and II) obeys the following equations (Borzani and Vairo, 1959):

Sacc. cerevisiae: $C/(C_i - C_f) = 0{\cdot}0114 + 0{\cdot}0915(1/C_f)$

S. lutea: $C/(C_i - C_f) = 0{\cdot}00432 + 0{\cdot}0287(1/C_f)$

The area covered by a molecule of crystal violet is $69{\cdot}0 \times 10^{-16}$ cm^2 (Brunauer, 1943), i.e., $S = 1{\cdot}02 \times 10^4$ cm^2/mg.

The specific areas of both micro-organisms can now be calculated:

Sacc. cerevisiae: $\sigma_1' = 90 \times 10^4$ cm^2/g dry matter

S. lutea: $\sigma_2' = 236 \times 10^4$ cm^2/g dry matter.

Discussion

The adsorption method proposed in this paper is based on the hypothesis that uniform unimolecular layers are formed when certain dyes are adsorbed by micro-organisms. This hypothesis was not tested directly; but when the Langmuir isotherm is followed, the unimolecular layer of adsorbed material on the adsorbent surface presents, with a high probability, a good picture of the phenomenon.

The examples presented in this article show that the values of σ and s determined by the adsorption method are greater than those calculated from direct measurements. This fact supports the validity of the proposed method, since certainly the true values of σ and s must be greater than the corresponding values calculated from microscopic measurements.

The results presented in Examples I, II, and III, using different dyes for the same micro-organism, seem to be comparable, especially as different samples of the same micro-organism were used with methylene blue and with crystal violet. It can also be observed that the relations between the specific areas for *Sarcina lutea* and *Saccharomyces cerevisiae* are practically equal when measured with methylene blue or with crystal violet; in fact, $\sigma_2/\sigma_1 = 2{\cdot}1$ and $\sigma_2'/\sigma_1' = 2{\cdot}6$.

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Redox Potential Changes in the 2-Keto-L-gulonic Acid Fermentation—I. Correlation between Redox Potential and Dissolved-Oxygen Concentration*

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Summary. The redox potential changes in the oxidative fermentation of L-sorbose to 2-keto-L-gulonic acid were investigated, using a mutant strain of *Pseudomonas*. The growth medium contained sorbose as the sole carbohydrate source. It was found that the redox potential curve of the fermenting system indicated qualitatively the existing oxygen demand of the culture. Increasing aeration rates led to higher oxygen demand and lower redox potentials. A comparison of the redox potential curves revealed that although the amount of dissolved oxygen directly affected the respiration of the culture, it did not necessarily mean respiratory insufficiency. The redox potential difference between the aerated and the deoxygenated (nitrogen flushed) fermentation broth was found to be linearly proportional to the logarithm of the dissolved-oxygen concentration. This observation served as the basis of a new quantitative method of dissolved-oxygen measurement, for which the name differential redoxymetry was proposed.

Introduction

The formation of 2-keto-L-gulonic acid (KGA) results from the microbiological oxidation of L-sorbose by a mutant strain of *Pseudomonas*, involving C-1 of the sorbose molecule. This oxidation is different from other known *Pseudomonas* oxidations, where hexonic acids or hexols are oxidized on C-2 or C-5 in a ketogenic oxidation (Narrod and Wood, 1956), or aldohexoses are oxidized on C-1 in an acidogenic (carboxyl-forming) oxidation (Entner and Doudoroff, 1952). The KGA oxidative fermentation process is

* Data presented in this paper were taken from a thesis, submitted in June, 1961, to St. John's University, New York, as partial fulfilment of the requirement for the degree of Doctor of Philosophy.

strongly aerobic, requiring a high amount of oxygen. A knowledge of the amount of dissolved oxygen (DO) is therefore crucial in this fermentation. The known methods of determining DO in fermentation broth (Finn, 1954) are somewhat cumbersome and frequently indirect; further they do not give information regarding the metabolic state of the oxidizing enzyme system. A knowledge of the redox potential of the system can be useful in aeration efficiency studies. The redox potential represents the overall tendency of the system to receive or donate electrons, i.e. it represents the reducing or oxidizing tendency of the system (Hewitt, 1950; Clark, 1960). Squires and Hosler (1958) found a linear relationship to exist between the redox potential and the logarithm of DO concentration in an uninoculated streptomycin fermentation medium, where different DO levels were established by using gas mixtures with different partial pressures of oxygen.

In this study, the usefulness of the redox potential for direct DO measurement and for oxygen-demand estimations was investigated in the 2-keto-L-gulonic acid fermentation, employing the mutant strain of *Pseudomonas*.

Materials and Methods

Preparation of the inoculum. The culture, an ultraviolet-irradiated mutant strain of an unidentified species of *Pseudomonas* consisted of Gram-negative, rod-shaped cells, approximately 4–8 μ long. The cells occurred mostly in chains, giving the culture a filamentous appearance. The culture was maintained on Emerson agar slants at 4°C, and was transferred bi-weekly.

Inoculum was prepared in the following medium: glycerol, 1·0 g; corn-steep liquor, 20 g; soy flour, 5·0 g; KH_2PO_4, 0·7 g; $MgSO_4 \cdot 7H_2O$, 0·2 g; $FeSO_4 \cdot 7H_2O$, 0·1 g; and water to 1 l.; pH 7·0. Fifty ml of this medium was inoculated with a 2 per cent volume cell suspension washed from a slant. The inoculated medium was incubated on a rotary shaker (120 rev/min) at 28°C for 24 h.

Fermentation equipment and medium. The fermentation experiments were conducted in 14-l. agitated, glass laboratory fermentors (New Brunswick Scientific Co.) each containing 10 l. of the

medium. The fermentation medium consisted of the following:

	g/l.
glycerol	1·0
L-sorbose	20·0
corn-steep liquor*	10·0
NZ amine B†	5·0
$K_2HPO_4 \cdot 3H_2O$	0·7
KH_2PO_4	0·3
NaCl	0·5
$MgSO_4 \cdot 7H_2O$	0·1
$FeSO_4 \cdot 7H_2O$	0·1

The fermentors were inoculated with a 2 per cent cell suspension obtained from the inoculum medium. Fermentations were conducted at 28°C. The pH of the medium was 7·5 before sterilization and 7·0–7·2 after sterilization. The pH was adjusted with NH_4OH, whenever necessary. Dow silicone oil (0·25 g/l.) was used as antifoam in the fermentors.

Redox potential measurement. For potential measurement, standard-size Beckman platinum inlay electrodes and specially constructed platinum sphere electrodes were used against a saturated calomel electrode. A Beckman Zeromatic pH meter was used as the recording instrument. The electrodes were permanently inserted in the fermentor and were sterilized with it. The arrangement can be seen in Fig. 1. In the fermentation

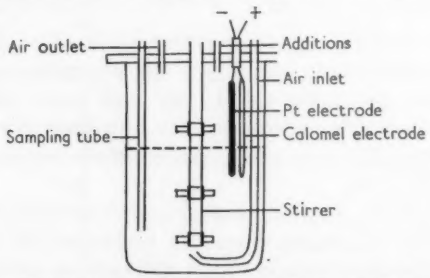

Fig. 1. Redox electrode arrangement in laboratory fermentor

experiments, only potential differences were needed, and consequently it was sufficient to regard the observed potential of the air-saturated medium as zero, with subsequent readings giving ΔE

* Corn Products Co.; 22–24 per cent protein on a wet basis.
† Casein hydrolysate: Sheffield Co.; total N_2: 12.7 per cent.

directly. Two kinds of ΔE measurements appear in this study: one is the direct measurement in the presence of oxygen, designated as ΔE_{O_2}, the other is the measurement in an N_2-flushed sample, designated as ΔE_{N_2}. The difference:

$$\Delta E_h = \Delta E_{O_2} - \Delta E_{N_2}$$

can be used as a measure of DO.

Oxygen-transfer measurement. For standard DO measurement the voltammetric method first described by Lewis and McKenzie (1947) and later applied by Hixson and Gaden (1950) was used. A recording polarograph (Sargent Model XXI) was used for the measurements. A single potential of $-0{\cdot}5$ V was selected between the dropping mercury electrode and the saturated calomel electrode. From the slope of the chart curve of the polarograph, the oxygen demand rate dc_L/dt was obtained. The oxygen absorption coefficient K_La and the specific respiration rate were calculated from the equations (Finn, 1954):

$$\frac{dc_L}{dt} = K_La(c_i - c_L) \tag{1}$$

and

$$\frac{dc_L}{dt} = c_C Q_{O_2} \tag{2}$$

were c_L is the actual oxygen concentration in g/l., c_i is the maximum oxygen solubility in g/l., K_La is the absorption coefficient in g O_2 absorbed per hour per l. per unit conc. difference in g/l.; Q_{O_2} is the specific respiration rate in g O_2/g dry weight per hour; t is the time in hours; and c_C is the cell concentration in g dry weight per l.

Assay procedures. Growth was measured turbidimetrically and dry weight was calculated from the turbidimetric readings by means of a calibration curve. KGA and sorbose were assayed by descending paper chromatography, using Whatman paper, No. 4. The solvent system consisted of methyl ethyl ketone: *n*-propanol: 3 per cent aqueous NH_3 (4:2:2). Running time was 30 min. The R_f for KGA as well as for 2-keto-D-gluconic acid was found to be 0·22. The 5-keto hexonic acids gave an R_f value of 0·5–0·6. For the determination of KGA, the dried chromatogram was

dipped in a reagent consisting of 0·2 per cent *o*-phenylene diamine · HCl in 95 per cent alcohol, containing 1 per cent HNO_3. The paper was heat-treated at 70 °C and KGA was detected as a bright yellow spot under a low intensity mercury light. For the determination of L-sorbose, the following reagent was employed : 40 mg bromocresol purple plus 100 mg boric acid dissolved in 100 ml methanol, to which 7·5 ml of 1 per cent borax solution was added. After dipping and heat treatment, sorbose was detected as a yellow spot against a blue background. KGA was isolated in pure crystalline form according to the procedure of Matasaka (1953).

Data were compiled from repeated runs employing duplicate or triplicate experiments. Only typical results were considered in the compilation.

Results

Fig. 2 and Table I show the results of a typical KGA fermentation. The fermentor was aerated at a rate of 1·0 v/v per minute,

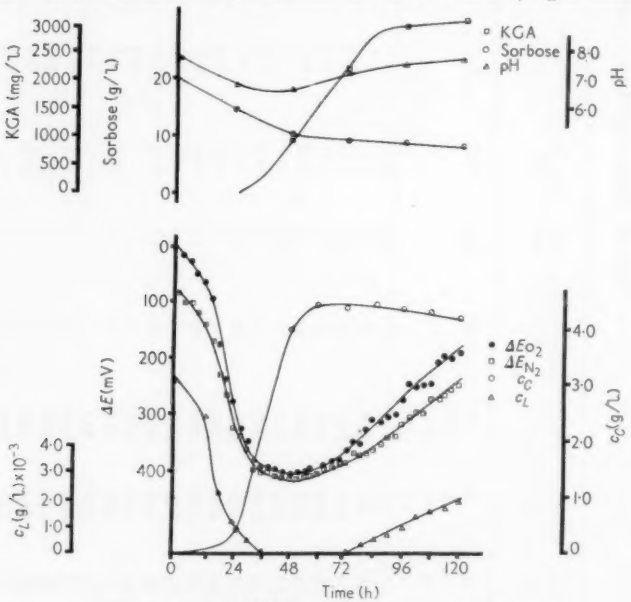

Fig. 2. Typical KGA fermentation parameters

Table I. Typical KGA fermentation parameters

Time, h	ΔE_{O_2}, mV	ΔE_{N_2}, mV	$\Delta E_{O_2} - \Delta E_{N_2}$, mV	D, mm	$c_L \times 10^{-3}$, g/l.	$c_i \times 10^{-3}$, g/l.	$K_L a$, l./h	KGA, mg/l.	S, g/l.	pH	c_C, g/l.	$\frac{dc_L}{dt} \times 10^{-2}$, g/l. h
0	0	82	82	65	6·0	6·2		0	20	7·8		
3	15	100										
6	25	100										
9	50	120										
12	65	140	77	44	4·0	6·2	22·0					2·6
15	95	170										
18	175	230	60	26	2·2	6·2	23·5					9·4
21	240	265										
24	275	325	46	15	1·2	6·2	22·5	0	14	6·8	·36	11·3
27	325	335										
30	350	365	10	9	0·5	6·3	24·0					13·9
33	385	400										
36	400	405	5	4	0·1	6·3	25					15·5
39	400	400										
42	405	410	5	–	–	6·3	25					15·8
45	410	415										
48	410	415	5	–	–	6·3	25	900	10	6·7	4·00	15·8
51	405	415										
54	400	412	5	–	–	6·3	25					15·8
57	400	410										
60	400	405	10	–	–	6·3	25				4·45	15·8
63	395	400										
66	385	395	15	–	–	6·3	25					15·8

69	380	390										
72	365	390	25	5	0·1	6·3	25	2100	8	7·25	4·40	15·5
75	350	370										
78	345	375	25	7	0·3	6·3	25					15·0
81	325	370										
84	315	365	30	9	0·45	6·3	25				4·45	14·6
87	315	355										
90	305	345	40	11	0·7	6·2	25					13·8
93	300	340										
96	275	320	45	13	0·9	6·2	25	3000	7	7·5	4·35	13·3
99	245	310										
102	250	300	54	17	1·3	6·2	25					12·2
105	250	300										
108	245	275	56	19	1·5	7·2	25				4·27	11·8
111	210	275										
114	200	270	58	20	1·6	6·3	25					11·5
117	200	260										
120	190	250	60	23	1·9	6·2	25	3150	6	7·5	4·18	10·7

and was stirred at 350 rev/min. The initial E_h of the air-saturated medium was 325 mV. In the calculations of K_La, c_L values below $0{\cdot}1 \times 10^{-3}$ were regarded as zero because of the inaccuracy of the measurement. The oxygen absorption rate was calculated from the equation:

$$\frac{dc_L}{dt} = K_L a c_i$$

where dc_L/dt was determined by saturating the sample with oxygen and following its disappearance voltammetrically. The slope of the curve gave dc_L/dt.

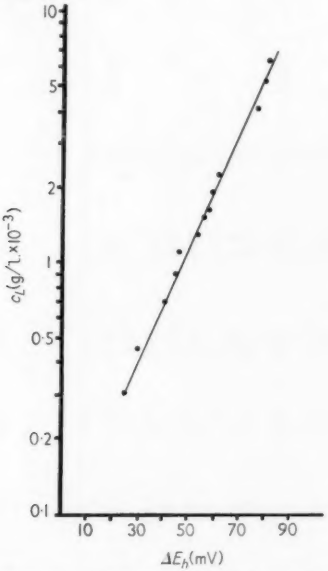

Fig. 3. Correlation between redox potential and dissolved oxygen concentration

Of the two redox curves, one was taken directly in the fermentor (ΔE_{O_2}), and the other with samples flushed with N_2 (ΔE_{N_2}). The potential difference between the two redox potential values, taken from adjusted redox curves, was plotted against the corresponding c_L values on semilogarithmic paper (Fig. 3), and a linear relationship was obtained. Fig. 4 and Table II show the effect of different aeration rates on the redox curve. This experiment was con-

Table II. K_La and Q_{O_2} calculations from the data of Table I

Time, h	Air, v/v	K_La, l./h	$c_L \times 10^{-3}$, g/l.	$(c_i - c_L) \times 10^{-3}$, g/l.[a]	$dc_L/dt \times 10^{-2}$, g/l. h	Q_{O_2}, g/g h	c_C, g/l.
12	0·5	22	4·8	1·4	3·1	8·6	0·36
24		22	1·2	5·0	12·0	8·4	1·30
48		25	–	6·2	15·1	4·3	3·60
72		25	0·1	6·1	15·0	3·4	4·50
96		25	1·3	4·9	12·2	3·2	3·80
12	1·0	23	4·9	1·3	3·0	8·3	0·36
24		24	1·7	4·5	10·8	8·0	1·35
48		25	–	6·2	15·1	4·5	3·45
72		25	–	6·2	15·1	3·4	4·60
96		25	1·4	4·8	12·0	3·1	3·90
12	1·5	27	5·0	1·2	3·2	8·1	0·40
24		28	1·9	4·3	12·0	8·0	1·50
48		29	0·1	6·1	17·5	4·7	3·70
72		29	0·1	6·1	17·5	3·8	4·60
96		29	1·5	4·7	14·6	3·2	4·20
12	2·0	31	5·1	1·1	3·4	8·5	0·40
24		31	2·2	4·0	12·4	8·0	1·60
48		32	0·2	6·0	19·2	4·8	4·00
72		31	0·3	5·9	18·3	3·9	4·70
96		31	1·9	4·3	13·3	3·2	4·20

[a] $c_i = 6{\cdot}2 \times 10^{-3}$ g/l.

ducted under the same conditions as the previous one, except that the aeration rate was varied between 0·5–2·0 v/v per minute. The

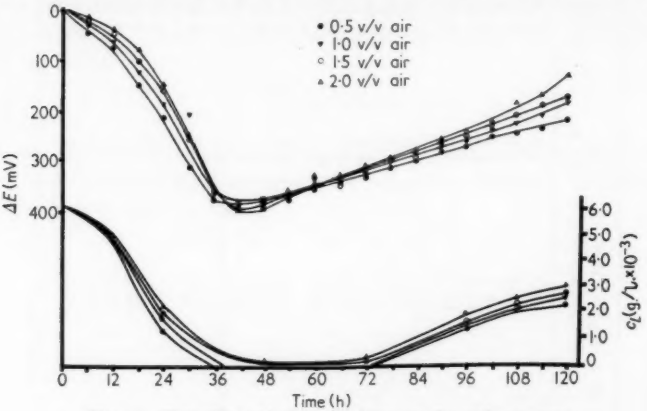

Fig. 4. The effect of air variations on the redox curve

variation in the oxygen absorption rate was relatively narrow, because of the single sparger aeration device of the fermentor. To determine the efficiency of the aeration, the critical DO level, c_{crit} (above which the oxidative metabolism of the culture should

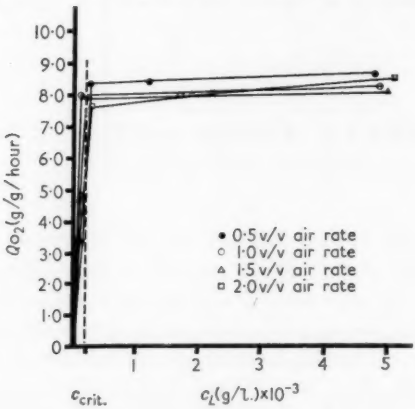

Fig. 5. c_{crit} determination graphically

be independent of the DO level), was graphically determined according to the method of Hixson and Gaden (1950) (Fig. 5). A figure of $0{\cdot}2 \times 10^{-3}$ g/l. was obtained as the most probable value for c_{crit}.

Discussion

A comparison of the redox curves and the c_L values in Tables I and II and Figs. 2 and 4 shows that the redox curve qualitatively characterizes the existing oxygen demand of the culture. The highest oxygen demand approximately coincided with the redox minimum and also with the c_L minimum and the highest growth rate. The strongly negative redox potential of the fermentation system represents an overall excess of electrons, which exists temporarily prior to their transfer to a suitable acceptor. The final acceptor being oxygen in this system, a negative redox potential truly represents a strong demand for oxygen.

Fig. 4 and Table II show that increased aeration leads to a lower redox minimum and a higher oxygen demand. This fact probably indicates that the amount of DO directly affects the metabolism of the organism; and the metabolism, in turn, affects the redox potential and the oxygen demand. The increased cell population in the increasingly aerated fermentors supports this view. In the experiment represented in Fig. 4, c_L was below the critical level in all but the most strongly aerated fermentors at the time of the redox minimum. Below c_{crit} the respiration rate (oxygen demand) should depend on the DO level, because of the unsaturation of the respiratory enzyme systems with oxygen (Winzler, 1941). This means respiratory insufficiency. The growth curves and KGA production curves of Fig. 4, however, do not indicate respiratory insufficiency, even at the lowest aeration rate employed. The calculated specific respiration rates in Table II show a declining tendency as the fermentation proceeds, but this is probably due to the non-homogeneity of the culture. The specific respiration rate data which served as a basis for the determination of c_{crit} were not obtained from a homogeneous culture. The fermenting culture is a mixture of young, aged and even dead cells. The respiratory requirements of these cells differ significantly, so that the measured specific respiration rate reflects only the overall

respiratory activity of the culture. Therefore, although the overall specific respiration rate decreases as the fermentation proceeds, the redox potential remains low. This indicates a normal respiratory activity, presumably by the action of the vigorous young cells of the population. It is possible that a delayed oxygen diffusion might also contribute to the lowering of the respiration rates (Longmuir, 1954). The normal increase in the total population also indicates that metabolic and respiratory activities are not inhibited by lack of oxygen. The same conclusion can be drawn from the fact that KGA production increased slightly as the aeration rate increased. In complex fermentation systems, the mechanical interpretation of c_{crit} is inadequate and might lead to false results in determining the aeration efficiency. A knowledge of the redox curves helps to clarify the situation in such cases, because it indicates the normality or abnormality of the oxidative metabolism of the culture.

The linear logarithmic relationship between DO and redox potential offers a simple new method for DO measurement. Because the method is based on the measurement of redox potential differences in the aerated and deoxygenated fermentation broth, the name differential redoxymetry is proposed for it.

Some limitations of the method should be pointed out. At low DO levels, below $c_L = 0{\cdot}5 \times 10^{-3}$, the method is not sensitive enough to give reliable results. There is great individual variation in the measured ΔE_{O_2}, and especially ΔE_{N_2}, values. A possible hypothetical explanation can be offered for the cause of these variations. At very low oxygen levels, the biological and colloidal chemical factors of oxygen absorption and desorption come to the fore. In a highly complex biological system, the physicochemical characteristics of this absorption and desorption are very complicated and far removed from the equilibrium state. On the other hand, this oxygen is part of a highly posed enzyme system at various stages of oxidation. In this system a sudden change in the oxygen level (by degassing the sample with nitrogen) shifts the balance of the overall redox potential. These factors are negligible at higher oxygen concentrations, but can be responsible for individual potential variations at low levels.

Keeping in mind the limitations, and using a careful statistical evaluation of the observed data, a valuable method is at hand for

the quick measurement of DO levels in actual KGA fermentations. The main advantage of the method, besides its simplicity, is the fact that it gives two important pieces of information at the same time:

1. The redox curve itself indicates the existing oxygen demand of the culture.

2. The difference of the two redox curves (ΔE_{O_2} and ΔE_{N_2}) indicates the amount of oxygen available.

Acknowledgements. The author thanks Dr. M. A. Pisano for his valuable help and encouragement, and Chas. Pfizer & Co. for the use of technical facilities.

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Redox Potential Changes in the 2-Keto-L-gulonic Acid Fermentation—II. Relationship between Redox Potential and Product Formation*

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Summary. In the investigation of the redox potential changes of the oxidative fermentation of L-sorbose to 2-keto-L-gulonic acid, it was found that the 2-keto-L-gulonic acid formation proceeded favourably at low negative redox potential values. Low redox potentials were maintained in a continuous fermentation process in the chemostat, with a resulting high 2-keto-L-gulonic acid formation rate. L-Tyrosine and some of its analogues stimulated the 2-keto-L-gulonic acid formation. The stimulation seemed to be bound to low redox potential values, and suggested the action of a direct oxidase-type enzyme.

Introduction

In the previous paper (Tengerdy, 1961), the relationship between redox potential and dissolved-oxygen concentration was studied in the oxidative fermentation of L-sorbose to 2-keto-L-gulonic acid by a mutant strain of *Pseudomonas*. It was found that the redox potential indicated the existing oxygen demand of the culture. The peak oxygen demand corresponded to the redox minimum, the most negative redox potential value of the culture.

The strongly negative redox potential reveals the reducing state of the complex redox systems of the fermentation broth ; the reducing state of the respiratory enzyme systems signifies strong metabolic activity. Therefore, some microbiological biosynthetic reactions proceed favourably at negative redox potentials, near the redox minimum of the redox curve of the particular culture involved. This was found to be true in the citric acid (Hewitt, 1950; Matkovics and Kovacs, 1957) and in the riboflavine fermentations (Kramli and Szabo, 1956).

* Data presented in this paper were taken from a thesis, submitted in June, 1961, to St. John's University, New York, as partial fulfilment of the requirement for the degree of Doctor of Philosophy.

In this study, the relationship between the redox potential and the 2-keto-L-gulonic acid formation was investigated.

Materials and Methods

Inoculum preparation, inoculation procedures and assays were made as described previously (Tengerdy, 1961). Fermentations were conducted in 300-ml Erlenmeyer flasks with 25 ml of medium, and in the chemostat described by Novick and Szilard (1951). The fermentation temperature was 28°C.

In the flasks, the following medium was used: glycerol, 1·0 g/l.; L-sorbose, 20·0 g/l.; L-asparagine, 10·0 g/l.; $K_2HPO_4 \cdot 3H_2O$, 0·7 g/l.; KH_2PO_4, 0·3 g/l.; $MgSO_4 \cdot 7H_2O$, 0·1 g/l.; $FeSO_4 \cdot 7H_2O$, 0·1 g/l.; $CuSO_4 \cdot 5H_2O$, 0·01 g/l.; pH 7·5, before sterilization.

In the chemostat the following medium was used: glycerol, 1·0 g/l.; L-sorbose, 20·0 g/l.; NZ amine B*, 10·0 g/l.; Bacto yeast extract, 0·5 g/l.; $K_2HPO_4 \cdot 3H_2O$, 0·7 g/l.; KH_2PO_4, 0·3 g/l.; $MgSO_4 \cdot 7H_2O$, 0·1 g/l.; $FeSO_4 \cdot 7H_2O$, 0·1 g/l.; $CuSO_4 \cdot 5H_2O$, 0·01 g/l.; pH 7·5, before sterilization.

The phenolic compounds employed in the experiment were added aseptically to the flask medium, after sterilization, from aqueous or alcoholic solution.

For redox potential measurement, thin platinum sphere electrodes and saturated calomel electrodes were used. The liquid connection between the fermentor and the calomel electrode was established by means of tygon tubing filled with a 3 per cent agar solution saturated with KCl. The platinum electrode and the tygon tubing were inserted permanently into the flasks or the growth tube of the chemostat, after sterilization (Figs. 1 and 2). Before measuring redox potentials, the potential of the calomel electrode plus bridge was established by using the platinum electrode as a Pt/quinhydrone electrode. In the actual fermentations, potential differences were measured directly.

The aeration rate was fixed in the flasks by keeping the volume constant (25 ml) throughout the experiments. In the chemostat the aeration rate was fixed by bubble count: 10 bubbles/sec were fed into the growth tube (about 13–15 ml air/15 ml medium/

* Casein hydrolyzate from Sheffield Co. Total N: 12·7 per cent.

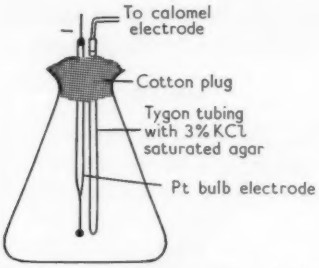

Fig. 1. Redox electrode arrangement in shake-flask

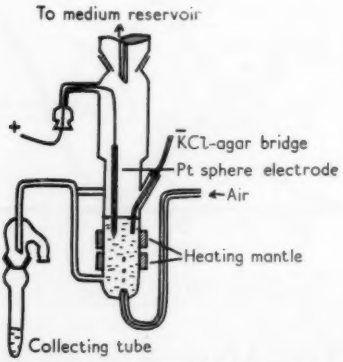

Fig. 2. The growth tube of the chemostat

minute). The K_La values were predetermined for the flasks and the chemostat by the sulphite method (Cooper, Fernstrom and Miller, 1944). A K_La of 24 was obtained for the flasks, and K_La = 17 was obtained for the chemostat.

The steady state in the chemostat was established by selecting a wash-out rate (W/V) which was equal to the mean bacterial division rate (Novick, 1955):

$$X = W/V$$

The wash-out, W, was 5 ml/h, the volume, V, of the growth tube was 15 ml, therefore:

$$X = \frac{5}{15} = 0{\cdot}33$$

(For comparison, in batch fermentation the highest calculated X was approximately 0·22.)

Results

A continuous KGA fermentation was conducted in the chemostat for 8 days. The results of the experiment can be seen in Fig. 3. The calculated KGA formation rate was high: a maxi-

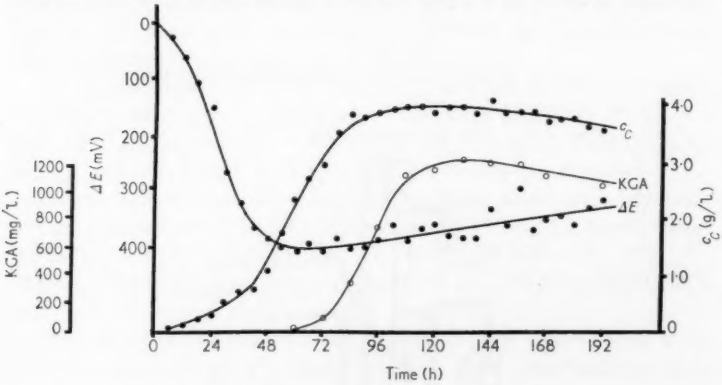

Fig. 3. Continuous KGA fermentation in the chemostat

mum rate of 396 mg/l. h KGA was recorded between 120 and 150 h ($0{\cdot}33 \times 1{,}200 = 396$). In the control batch process, the maximum KGA formation rate was 45 mg/l. h between 45 and 72 h.

Media studies with the *Pseudomonas* strain revealed the interesting observation that L-tyrosine increased KGA production significantly if added to a glycerol–L-sorbose–L-asparagine-type semi-synthetic medium. The effect was less marked or absent in natural media. This observation led to the hypothesis that a tyrosinase-like enzyme might be involved in the oxidation, and that the presence of the substrate (L-tyrosine) might stimulate enzyme formation.

This hypothetical enzyme induction was investigated in shake-flask experiments with L-tyrosine and some of its structural analogues. The compounds were employed at the maximum concentration tolerated by the organism. The results of the

experiment can be seen in Fig. 4. Only those compounds are shown which had a positive effect on the KGA formation.

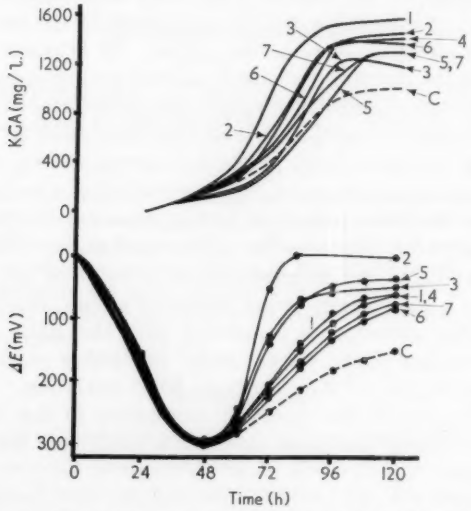

Fig. 4. The effect of phenolic compounds on the KGA fermentation
1. L-Tyrosine 1·0 g/l. 2. DL-Phenylalanine 1·0 g/l. 3. DL-*p*-Hydroxy phenyl-glycine 1·0 g/l. 4. *p*-Hydroxy benzoic acid 1·0 g/l. 5. *p*-Hydroxy phenyl acetic acid 1·0 g/l. 6. *p*-Amino-phenyl ethanol 0·5 g/l. 7. *p*-Cresol 0·2 g/l. C. Control.

Discussion

It was apparent from previous experiments (Tengerdy, 1961) that KGA formation started only when the redox minimum had been attained, and continued while the redox potential remained low (negative).

The results obtained show a significant correlation between the redox potential and the KGA formation. It is possible that the biosynthesis of the oxidizing enzyme takes place in the low redox potential region; this is conceivable if we consider the strong overall metabolic activity of the culture in this period. In this case, the formation of the enzyme and the enzyme concentration would be the limiting factors in the reaction. The equilibrium potential of the pure L-sorbose–KGA oxidation system itself is in

the positive potential region, but from the point of view of the reaction the redox potential does not seem to have any significance.

The effect of L-tyrosine and its analogues on the reaction also suggests an effect on enzyme formation. It is possible that L-tyrosine and the structurally closely-related analogues might act as enzyme inducers, thereby stimulating the KGA formation. If this enzyme induction takes place, it should take place at low redox potentials in the most likely period for substantial enzyme formation. Careful examination of Fig. 4 reveals that a sudden change appeared in the redox potentials in the presence of L-tyrosine and its analogues after reaching the redox minimum. Whether the effect of L-tyrosine and its analogues is inducive or not, the effect seems to be bound to low redox potential values, indicating that the low redox potential is connected with the KGA formation. This observation might prove useful in further studies on the reaction mechanism of the L-sorbose–KGA oxidation. The practical significance of the observed correlation is that it offers a means of attaining the most favourable conditions for the KGA fermentation.

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Measurement of Dissolved Oxygen in Fermentations*

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Summary. Two methods have been investigated for the measurement of dissolved oxygen in fermentations: the tubing method and the probe method. The sensing elements for both methods are steam-sterilizable and measure only the oxygen tension in the liquid phase. The tubing method is probably the most accurate and reliable method available for measurement of dissolved oxygen in fermentations but it requires extensive ancillary apparatus.

Introduction

In aerobic fermentations, the oxygen available to microorganisms is only that dissolved in the liquid phase and, until recently, good methods for measurement of this important fermentation variable were not available. Solid microelectrodes[4, 7] and dropping mercury electrodes[1, 8] have been used, but polarization of the electrodes, change in calibration with fermentation age, and interference from medium constituents have made measurement with these devices very difficult.

Measurement of dissolved oxygen in actual fermentations presents many problems. The devices for measurement should be steam-sterilizable and should perform satisfactorily under the mechanical strain of operation in an agitated fermentor. Calibration should be independent of the chemical and physical properties of the fermentor contents and should be stable over a period of days.

Recently, Gualandi *et al.*[6] have described two devices for measurement and have presented dissolved-oxygen data on several

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fermentations. Carritt and Kanwisher[3] have also described an oxygen electrode that appears adaptable to measurement of dissolved oxygen in fermentations. With their device, and with the devices to be described here, oxygen is allowed to diffuse from an external phase (microbial culture), through an oxygen-permeable membrane and into an internal phase where the oxygen tension must be very low. This is shown diagrammatically in Fig. 1.

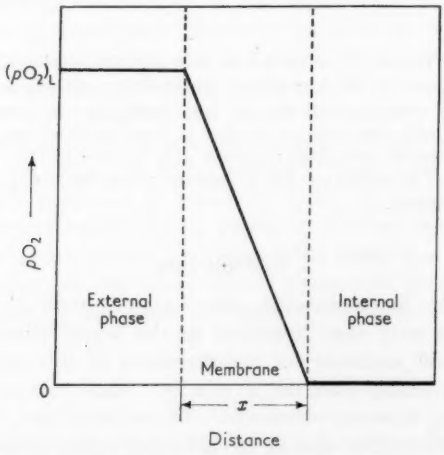

Fig. 1. Oxygen activity profile for diffusion through a membrane. The oxygen diffusion rate, N, through the membrane is $AD[d(pO_2)/dx]$ or $N=[AD(pO_2)_L/x]$ where A = area of the membrane, D = diffusivity of oxygen in the membrane, x = thickness of the membrane, and $(pO_2)_L$ = oxygen tension in the external phase

The rate of oxygen transfer is proportional to the oxygen activity gradient in the membrane and this is proportional to the oxygen tension in the external phase provided the oxygen tension in the internal phase is very low. Therefore, in order to use this method for measurement, an external phase must be separated from an internal phase with an oxygen-permeable membrane, the oxygen tension in the internal phase must be maintained at a low level, and it must be possible to measure some quantity proportional to the rate of oxygen transfer through the membrane.

Two methods have been investigated. Both use the above basic principle, but the methods for evaluation of the oxygen-

transfer rate across the membrane and for maintenance of negligible oxygen in the internal phase are different.

Results and Discussion

Tubing Method

The tubing method is illustrated in Fig. 2. A coil of Teflon tubing (the oxygen-permeable membrane) is immersed in the

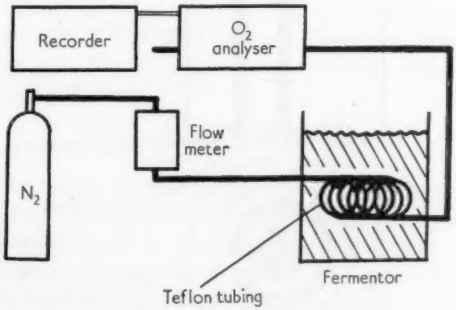

Fig. 2. Apparatus for the tubing method

fermentor. A slow stream of pure nitrogen is passed through the tubing at a constant rate and into a Beckman oxygen analyser. Some of the oxygen dissolved in the fermentation medium (the external phase) diffuses through the Teflon, and the oxygen content of the effluent nitrogen is proportional to the oxygen tension in the medium. The sensitivity of this method is dependent on the length, diameter and wall thickness of the tubing and on the nitrogen flow rate. With 40 ft of 1/8-in. diameter tubing of 0·012-in. wall thickness, the effluent gas was found to contain 0·3 per cent oxygen when the influent nitrogen flow rate was 20 ml/min through the tubing in air-saturated water at 30°C. With this design, the gas flow rate through the oxygen analyser was lower than the rate that was recommended by the manufacturer and the oxygen content of the effluent gas was too low to allow the desired sensitivity. Consequently, if this method were redesigned, a longer Teflon tube (about 100 ft) and a higher gas flow rate (about 40 ml/min) would be used.

Probe Method

An oxygen probe is shown diagrammatically in Fig. 3. The device is similar in principle to that described by Carritt and

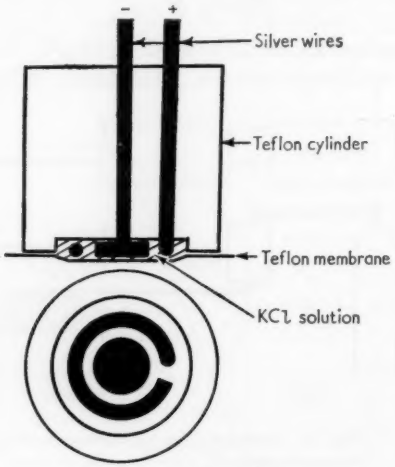

Fig. 3. Oxygen probe

Kanwisher.[3] In this method, oxygen diffuses from an external phase, through the membrane and into the internal phase where the oxygen is electrolytically reduced. The potential between the anode and cathode must be continuously applied so that the oxygen tension in the internal phase is very low. The current required for electrolysis is proportional to the oxygen tension in the external phase.

A number of membrane materials have been investigated for use in this method (Watanaba and Leonard, Beckman Instruments, Inc., Fullerton, California). Polyethylene, Teflon, polyvinyl chloride, natural rubber and silicone rubber possess sufficiently high oxygen diffusivities to allow their use, but Saran and Mylar were too impermeable. For steam-sterilizable probes, Teflon was used for the membrane material. Rubber membranes were investigated but these were too permeable to other substances.

We found that it was convenient to use silver for the anode and cathode and a KCl solution for the internal phase. The electrode reactions were

$$Cl^- + Ag^\circ \rightarrow AgCl + e^- \text{ (anode)}$$

$$\tfrac{1}{2}O_2 + H_2O + 2e^- \rightarrow 2OH^- \text{ (cathode)}$$

Both silver and Cl^- are used in the anode reaction. Therefore, the life of a probe is determined by the availability of these materials. In actual practice, it is easy to make the silver anode so large that silver does not become limiting. Therefore, Cl^- usually limits the life of a probe. However, when the Cl^- is exhausted, the following anode reaction occurs:

$$Ag^\circ + OH^- \rightarrow AgOH + e^-$$

Although this might appear to be a perfectly acceptable anode reaction, it has two serious disadvantages. First, silver oxide does not adhere strongly to the anode; it loosens and accumulates on the membrane, causing the sensitivity of the probe to decrease. Actually, this causes a resistance to oxygen diffusion in the internal phase. Secondly, the solubility of silver oxide is much greater than that of AgCl and, as a result, the residual current (the current when the oxygen tension in the external phase is zero) is much greater with the Ag–AgOH anode reaction because of reduction of silver. Probably the silver reduction current contributes significantly to the residual current and is controlled by the availability of silver ions at the cathode.

Obviously, the higher the Cl^- quantity in the internal phase, the longer the life of the probe. Consequently, several methods to increase the Cl^- quantity in the internal phase were investigated.

First, we attempted to use a solution that was saturated with KCl for the internal phase. However, AgCl forms a complex ion in the presence of high Cl^- concentrations and considerable silver electroplating occurred. This not only caused a high residual current but electroplating was so heavy that bridges of metallic silver formed between the anode and cathode, short-circuiting the probe. The optimum Cl^- concentration appeared to be about 0·75M.

Secondly, we attempted to use a solution buffered with respect to Cl^-. Ion-exchange resin and resin paper in the chloride form were tried, but repeated steam sterilization caused these materials to deteriorate and accumulate on the membrane, causing a diffusion barrier in the internal phase which resulted in a loss of sensitivity.

Thirdly, we attempted to increase the volume of the internal phase. Three hypothetical probes and the oxygen-activity gradients for each are shown in Fig. 4.

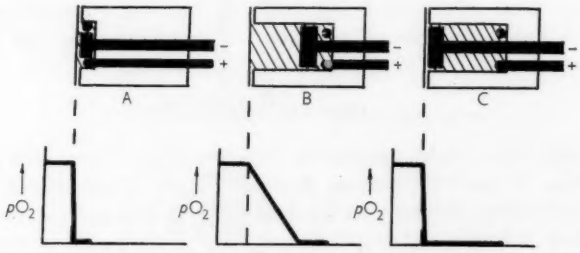

Fig. 4. Various oxygen-probe designs

In probe A, the volume of the internal phase is low and no gradients occur in the internal phase.

In probe B, the volume of the internal phase is large and the distance between the membrane and the cathode is so large that oxygen-activity gradients develop in the internal phase. These gradients usually present a serious problem. They require considerable time to form and disappear when the oxygen tension in the external phase changes. As a result, this probe would respond very slowly to changes in oxygen tension. The sensitivity of this probe would be much less than that of probe A because of the additional resistance to oxygen diffusion in the internal phase. Finally, the data recorded with probe B would be very erratic because mechanical vibration would cause erratic eddy diffusion in the internal phase.

In probe C, the volume of the internal phase is large, but no oxygen-activity gradients occur in the internal phase because the distance between the membrane and the cathode is small. Actually, this is a good design for non-steam-sterilizable probes, but

when this design was used for steam-sterilizable probes, thermal expansion of the large internal phase caused serious deformation of the membrane.

Design data for a steam-sterilizable probe are shown in Fig. 5. A small pool for the anode and cathode was machined in the end

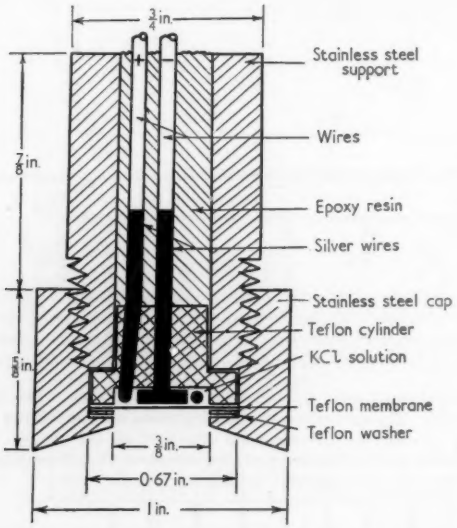

Fig. 5. Oxygen probe. Details of construction

of the Teflon cylinder. Two 0·040-in. holes were drilled longitudinally through the cylinder. The anode and cathode were forced through these holes after they were shaped from 0·050-in. silver wire. Insulated wires were soldered onto the upper end of the electrodes and the support was placed in the collar. The soldered connections were potted in epoxy resin. After the resin polymerized, the pool was filled with 0·75M KCl and a 0·005-in. Teflon membrane was placed over the pool. The cap, containing the small Teflon washer, was tightened onto the membrane. Particular care was taken to exclude all air bubbles from the KCl solution during this operation.

The electrical apparatus for operation of six probes is shown in Fig. 6. The same potential was applied to all of the probes. The current was recorded (as a voltage drop through a suitable resistor) on a six-point recording potentiometer with a 10-mV range.

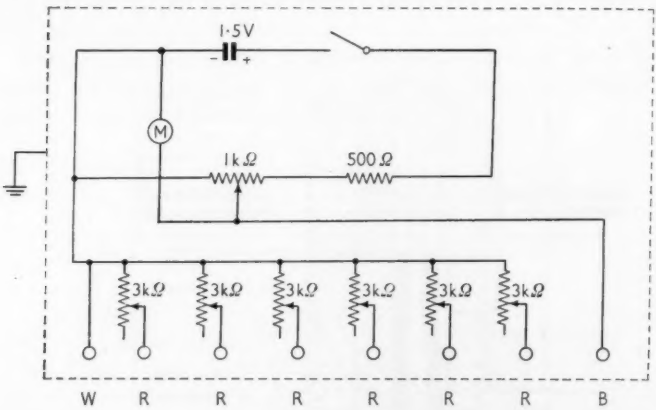

Fig. 6. Electrical apparatus for operation of six probes. The battery was a No. 6 ignition cell. The wires from the binding posts were as follows: W = common negative input of recorder, B = common anode of all six probes, R = individual cathodes of probes and individual positive inputs of 10-mV multipoint recorder, and M = voltmeter, 0–1 V

Before a probe can be used for measurement of dissolved oxygen, the oxygen must be exhausted from the internal phase. The time required for exhaustion of this oxygen will be called the warm-up time. A typical warm-up curve is shown in Fig. 7. When the potential was first applied to the probe, the current was very high, but it eventually reached a constant value. The warm-up time varies with electrode design. For probe A shown in Fig. 4, the warm-up time is several hours. For probe C shown in Fig. 4, it is over one day.

We found that it was most convenient to allow the probes to operate overnight with an applied potential of about 0·7 V before data for a polarogram were recorded. Crude data for a polarogram are shown in Fig. 8. Each time the potential was increased, an initial current surge was noted, but the current eventually

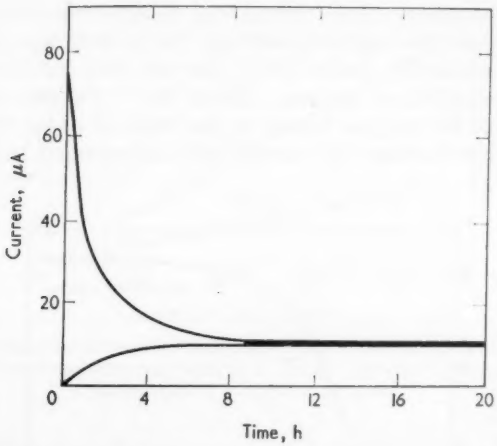

Fig. 7. Warm-up curve for oxygen probe. The upper curve gives the total observed current; the lower curve the current due to reduction of oxygen continuously diffusing through the membrane. The area between the curves represents the number of coulombs needed to reduce the oxygen originally present in the internal phase

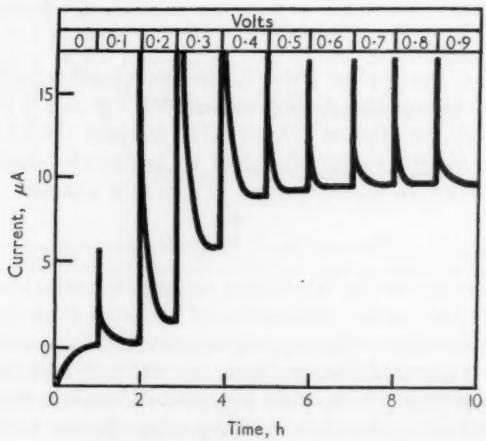

Fig. 8. Crude data for a polarogram

reached a constant value. When the steady-state current was plotted against the applied potential, the polarogram shown in Fig. 9 was obtained. Below 0·1 V, the potential was insufficient to cause reduction of oxygen. Above 0·5 V, oxygen reduction occurred and the oxygen tension in the internal phase was probably very low because the current was independent of voltage.

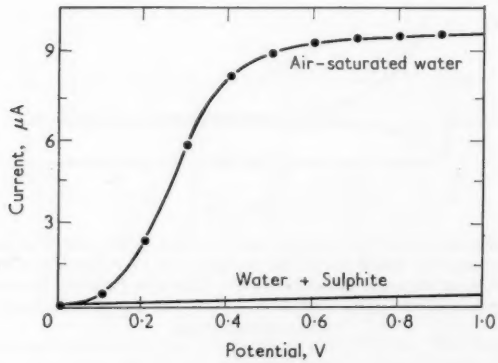

Fig. 9. Polarogram for oxygen probe. These results were obtained from the data shown in Fig. 8

Consequently, the applied potential for measurement of dissolved oxygen with this probe should be between 0·5 and 1·0 V. This potential must be applied continuously because the oxygen tension in the internal electrolyte must be essentially zero for good measurement of dissolved oxygen in external phases.

Evaluation of Methods

Calibration curves for the tubing and probe methods are shown in Fig. 10. The curves are essentially linear for both devices.

The response times of the tubing and probe are shown in Fig. 11. The probe responded faster than the tubing, but the tubing reached a constant value while the probe's current continued to decrease slightly. This slow decrease might be due to removal of oxygen-activity gradients from the internal phase.

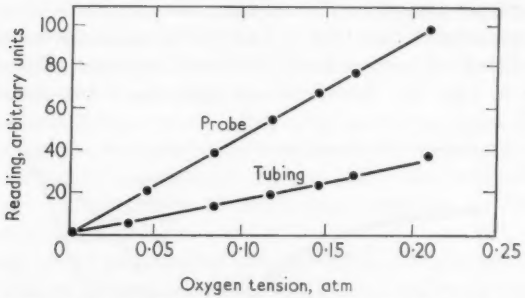

Fig. 10. Calibration curves for tubing and probe. The devices were placed in water at 30°C and the water was saturated with gas mixtures containing various percentages of oxygen in nitrogen

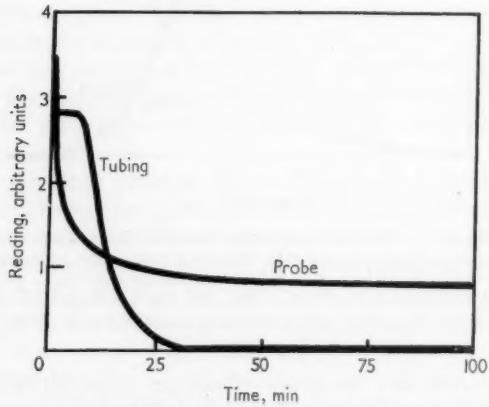

Fig. 11. Response curves for tubing and probe. The devices were placed in air-saturated water at 30°C, and at zero time sodium sulphite was added to the water

The calibration of the tubing was very stable. In fermentations, it was impossible to detect a change in calibration. However, calibration of the probes changed during a fermentation. The zero point was usually quite stable but the sensitivity decreased with fermentation age (or time of operation). This decrease was

as much as 20 per cent per day for new probes and as little as 10 per cent per week for older probes.

A comparison of the tubing and probe methods for measurement of dissolved oxygen in an *Aerobacter aerogenes* fermentation is shown in Fig. 12. Both devices gave about the same results.

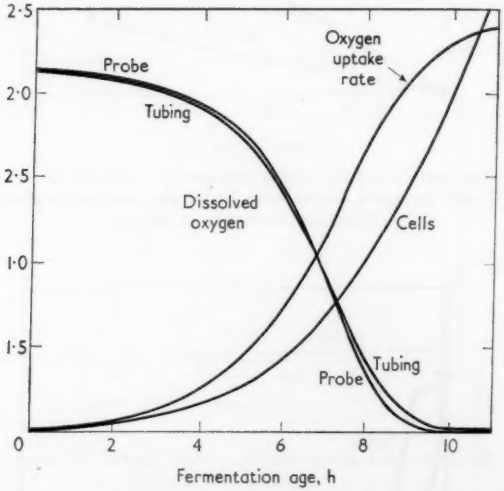

Fig. 12. Data on an *Aerobacter aerogenes* fermentation. Units are: dissolved oxygen, atm × 0·1; oxygen uptake rate, mmoles/l. min; cells, g (dry basis)/100 ml. The fermentation was performed in a 3·5-l. fermentor that had a sulphite oxidation value of 1·3 mmoles O_2/l. min. The pH was automatically controlled at 7·0 ± 0·1 and the temperature was controlled at 30°C

It will be noted that the dissolved-oxygen level fell to zero when the cell population became very high. The effluent gas from the fermentor contained about 10 per cent oxygen. Therefore, these devices must record only the oxygen dissolved in the liquid phase.

Calibration of Tubing and Probes

Before dissolved oxygen can be measured in fermentations, the measuring device must be calibrated. Since both the tubing and probe exhibit linear calibration curves (see Fig. 12), only two points

are necessary for construction of a calibration curve. Calibration of the probe is complicated by steam sterilization because during this process the internal electrolyte expands and the membrane characteristics change. Sterilization has practically no effect on the calibration of the tubing.

The air-saturation point (calibration point corresponding to a dissolved-oxygen tension of approximately 0·21 atm, at 1 atm of absolute pressure) can be easily obtained after sterilization on most fermentors. However, it is much more difficult to obtain the second calibration point.

The zero point (calibration point corresponding to no dissolved oxygen) can be determined in the laboratory before the measuring device is placed in the fermentor. This method is sometimes acceptable because sterilization has only a slight effect on the zero point.

In small equipment, the medium can be saturated with nitrogen after sterilization and the zero point can be determined. This method is usually impractical in large equipment.

Aeration can be stopped for a short period after inoculation, and after the micro-organisms have consumed all of the dissolved oxygen the zero point can be determined. This method has obvious disadvantages.

If the operating pressure of the fermentor is changed, it is possible to calculate the change in dissolved-oxygen tension. Consequently, the slope of the calibration curve can be calculated and if one calibration point is known, the curve can be constructed.

For the tubing method, the zero point corresponds to zero per cent oxygen in the effluent gas from the tubing. Therefore, the zero point is automatically established when the oxygen analyser is standardized.

At the end of the fermentation, it is often desirable to recalibrate the measuring devices. The zero point can be determined when aeration is discontinued because the micro-organisms very soon consume all of the dissolved oxygen. The air point can be determined by two methods. First, if the oxygen demand of the culture is negligible at the end of the fermentation the dissolved oxygen is approximately in equilibrium with the air and the approximate air point can be determined. Actually, it is usually practical to apply a small correction to this approximate value

based on the oxygen uptake rate of the culture, N, and the approximate absorption coefficient K_La. ($N = K_La[(pO_2)_G - (pO_2)_L]$, where $(pO_2)_G$ and $(pO_2)_L$ are the oxygen partial pressures in the gas and liquid phases, respectively.) Secondly the fermentor contents can be removed and the vessel can be refilled with air-saturated water. The air point can be determined because calibration (in pO_2 units) is independent of the nature of the external phase. It should be pointed out that calibration made at one temperature is not valid at a different temperature. Since most fermentations are carried out at a controlled temperature, the temperature coefficient of the measuring device can usually be neglected.

Conclusions

Two methods have been investigated for measurement of dissolved oxygen in fermentations. Both methods have certain advantages and disadvantages.

(1) The initial speed of response of the probe was much greater than that of the tubing. However, when the dissolved-oxygen tension was changed, the tubing reached a constant value before the probe.

(2) Although both devices are steam-sterilizable, fewer complications were encountered in sterilization of the tubing.

(3) The tubing method is more accurate than the probe method for measurement of dissolved oxygen in fermentations because calibration of the tubing is more practical and more stable.

(4) The tubing method requires a sensitive oxygen analyser while the probe method requires relatively simple ancillary apparatus.

(5) The life of an oxygen probe is about 5 days (for continuous operation in air-saturated liquid) while the tubing should last indefinitely. The KCl in the probe must be renewed about once a week.

(6) The probe method gives a time average of dissolved oxygen at one point while the tubing method gives a time and space average.

When it is desirable to measure dissolved oxygen in fermentations where high accuracy is not essential, the probe method is

probably satisfactory. This method is ideal for measurement at various points in a fermentor for design and evaluation purposes. However, for measurement in long fermentations or where high accuracy is essential, the tubing method is most desirable. This method appears to be well suited to measurement in continuous fermentations.

The requirements of a device for the control of dissolved oxygen are more stringent than those for measurement. Consequently, the tubing method may find considerable application in the control of dissolved oxygen in fermentations.

Both methods are practical and convenient for use in actual fermentations. Measurement of dissolved oxygen in the medium during fermentation makes possible direct evaluation of oxygen absorption coefficients under the conditions of the fermentation without reliance on artificial models such as sulphite solutions.[2, 5] Such direct evaluation should be of great value in the aeration problem involved in scale-up. In fact, it might prove advantageous to maintain the dissolved-oxygen tension in cultures at economically optimal levels instead of at the usually unknown levels resulting from the use of an arbitrarily selected method of operation.

Acknowledgement. The authors are grateful to David Freedman (New Brunswick Scientific Company) for several very helpful suggestions which led to the probe design shown in Fig. 5.

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Aeration in Fermentations

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Summary. Symptoms of oxygen deficiency and variations in dissolved-oxygen level have been described in some typical aerobic fermentations. In bacterial fermentations, dissolved-oxygen levels were almost the same in different parts of the fermentor. As a result, good correlations between apparent and true critical oxygen concentrations were obtained. Poor correlations were obtained in mould fermentations.

Introduction

In recent years, submerged-culture fermentation has become an extremely important industrial operation. Improved media and high-yielding strains have greatly increased the productivity of aerobic fermentors. However, in order to achieve high productivity, oxygen must be supplied at a rate that meets the needs of the micro-organisms.

The effect of dissolved oxygen on the metabolic rate of many cultures has been studied.[2, 12, 14, 18, 19] For most cultures, the critical oxygen tension (the highest oxygen tension at which the cells exhibit an oxygen deficiency) is below 0·02 atmospheres. Consequently, it can usually be concluded that oxygen supply in a fermentation is sufficient if the dissolved oxygen tension at every point in the fermentor is above 0·02 atm.

Considerable research has been devoted to the evaluation of aeration efficiency in fermentation vessels.[1, 4, 5, 8, 11, 16, 29, 32–34, 37] The most common method for this is the sulphite oxidation method.[7] In this method, the fermentor, which may be a shaken flask or a large tank, is filled with a copper-catalysed sulphite solution and the rate at which sulphite is oxidized to sulphate is used as a measure of aeration efficiency in the vessel. A good correlation between the oxygen uptake rate of sulphite solutions and the maximum rate in fermentations[2, 20, 27, 31] might be expected because the rate-controlling process appears to be the same

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in both systems.[17, 25] However, the maximum rate of oxygen uptake in both systems appears to be dependent on the physical properties of the solutions.[9, 21, 35] Therefore, a quantitative correlation would not be expected. Nevertheless, the sulphite method has been used successfully for fermentation scale-up,[3, 36] but occasionally poor results are obtained. If a quantitative correlation occurred between the oxygen uptake rate of sulphite solutions and the maximum rate in microbial cultures, the sulphite method might prove useful for evaluation of fermentor design variables. In our work, however, it was found that the degree of correlation depended on fermentor design, and could be very poor. Consequently, the sulphite oxidation method appears to have only limited application for evaluation of aeration in fermentors.

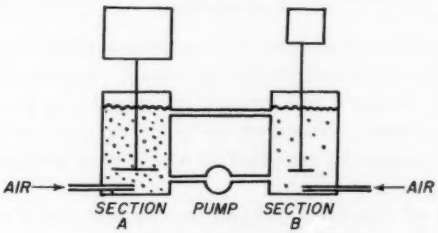

Fig. 1. Hypothetical fermentor

Some investigators[2, 22] have postulated that variations in dissolved oxygen might occur in fermentations, but only a little information on these variations is available.[13] Several years ago, we observed very large, short-time variations in dissolved oxygen at many locations in bacterial fermentations, but until recently did no further work on such variations. Data will be presented to show that variations in dissolved oxygen tension in fermentations can seriously affect the interpretation of dissolved oxygen measurements. Therefore, some common types of variations will be considered.

A hypothetical fermentor is shown in Fig. 1. This fermentor consists of two sections. Medium is circulated between them at a low rate. Section A has good aeration and section B has poor aeration (assume that the aeration efficiency in section A is five times greater than that in section B). Assume that this vessel is

inoculated with a culture whose critical oxygen tension is zero, that the cells are not injured by an oxygen deficiency, that the absorption coefficient is constant throughout the fermentation, and that the data shown in Fig. 2 are obtained.

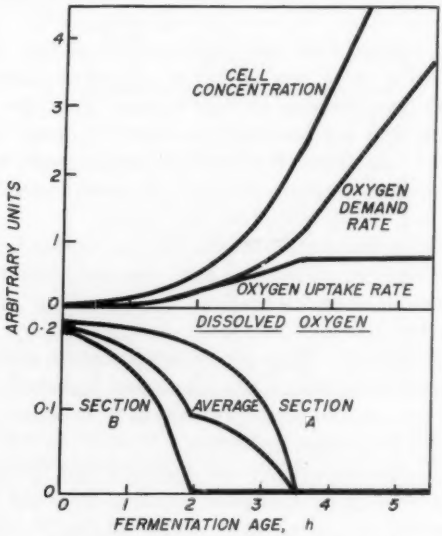

Fig. 2. Data on hypothetical fermentation

Shortly after inoculation, the oxygen tension in both sections is very high because the oxygen demand is very low. Sufficient oxygen is available and the oxygen demand and utilization rates are equal. As the population exponentially increases, the oxygen demand and uptake rates increase and the dissolved oxygen decreases. Eventually, the oxygen demand rate becomes so great that the dissolved-oxygen level in section B falls to zero. At this time, the fermentation begins to exhibit an oxygen deficiency. The cell concentration is no longer increasing exponentially and the oxygen demand begins to exceed the oxygen uptake rate because aeration is limiting metabolism. The population continues to increase, the oxygen demand continues to increase and the oxygen utilization rate continues to increase because aeration

is not limiting in section A. However, the dissolved-oxygen level in section A eventually falls to zero and the oxygen utilization rate becomes constant. Now the fermentor is operating at maximum aeration efficiency. The population increases linearly at a maximum rate after this time because aeration is limiting metabolism.

If dissolved oxygen were measured only in section A or if only the average value were recorded, the apparent critical oxygen tension (the oxygen tension at which signs of oxygen deficiency are first observed in a fermentation) would be much higher than the true value. However, if dissolved oxygen were measured in the region of lowest aeration (section B), good correlation would be obtained.

In this hypothetical fermentation, oxygen supply became limiting when the oxygen utilization rate was only one-third of the maximum possible rate. This had only a minor effect in this fermentation because the cells were not injured by the oxygen deficiency in section B. If an actual fermentation were performed with this hypothetical vessel, the maximum metabolic rate might be limited by the oxygen supply of section B, because cell injury is often caused by an oxygen deficiency.

Even though fermentors are not designed like the vessel shown in Fig. 1, regions of good and poor aeration do occur in some fermentors because of insufficient bulk mixing.[23] When these occur, the apparent critical oxygen tension may be much higher than the true value and the economically optimal metabolic rate in a fermentation may be limited by the region of lowest aeration.

Besides regional variations due to insufficient bulk mixing, short-time variations in dissolved oxygen also occur in fermentations. Bubble trails (the oxygen-rich liquid in the wake of air bubbles) account for the major short-time variations. Variations due to mycelial clumps[28] could not be demonstrated in our equipment.

Fig. 3 represents a small volume of medium in an agitated, sparged fermentation. When the oxygen utilization rate is very high, practically no oxygen is present in the bulk of the liquid. However, considerable oxygen is available in the wake of the bubbles because oxygen is continually diffusing from the gas phase

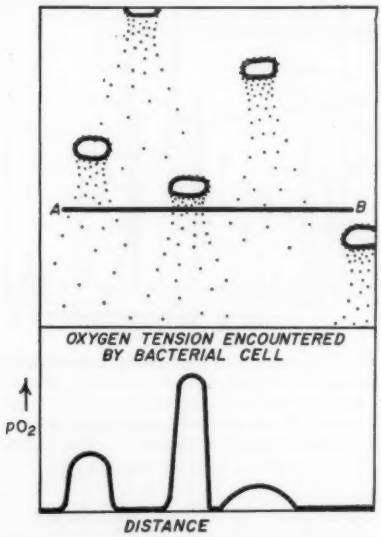

Fig. 3. Hypothetical volume in a fermentation. Assume that the fermentor is operating at maximum aeration efficiency. The dot density represents relative oxygen tension. The pO_2 encountered by a bacterial cell travelling from A to B is shown in the lower part of the figure

to the liquid phase. Consider a microbial cell moving from A to B on the line AB of Fig. 3. Shortly after the cell leaves A, it has excess oxygen because it is in the oxygen-rich trail of an air bubble. As the cell moves towards B, it encounters regions of very low oxygen tension when it is between bubbles and regions of excess oxygen when it is in the bubble trails. To show that these variations actually occur, a platinum microelectrode trace in an agitated, sparged, bacterial fermentation is shown in Fig. 4. These variations were not due to penetration of the gas bubbles by the electrode, because when this happened the current instantaneously decreased to zero because of lack of conductivity. If the time-average dissolved-oxygen tension, during the time of measurement shown in Fig. 4, were recorded, one might conclude that sufficient oxygen was available for aerobic metabolism. However, micro-organisms may not be able to traverse these regions of low oxygen

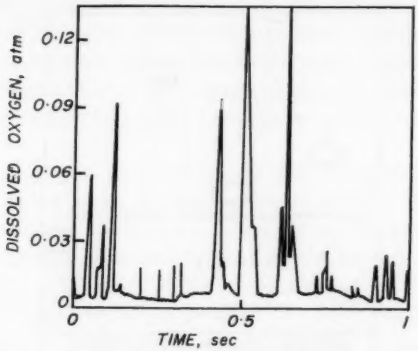

Fig. 4. Short-time variations in dissolved oxygen in an *Aerobacter aerogenes* fermentation. The fermentor had an operating volume of 500 ml and had very good aeration. The aeration efficiency as measured with the sulphite method was 4·9 mmoles O_2/l. min. These data were obtained with a platinum microelectrode (see Fig. 6) located near the impeller. The bubble trails were less numerous near the surface

tension without exhibiting an oxygen deficiency. Consequently, when time-average dissolved-oxygen tensions are measured in fermentations, the apparent critical oxygen tension may be higher than the true value.

Materials and Methods

Fermentation Analyses

The apparatus used to study aeration in fermentations is shown diagrammatically in Fig. 5. Air was passed through the fermentor at a constant, known rate. The oxygen and carbon dioxide contents of the effluent gas were measured with a Beckman oxygen analyser and a Liston–Becker carbon dioxide analyser, and recorded with a recording potentiometer. The probe method[26] was routinely used for measurement of dissolved oxygen. A recording potentiometer was also used for these measurements.

A stationary platinum microelectrode was developed for evaluation of the instantaneous value of dissolved oxygen at various points in a fermentor. The apparatus is shown in Fig. 6. A potential of about 0·4 V was applied between the electrode and the fermentor (which was grounded) and the current was measured

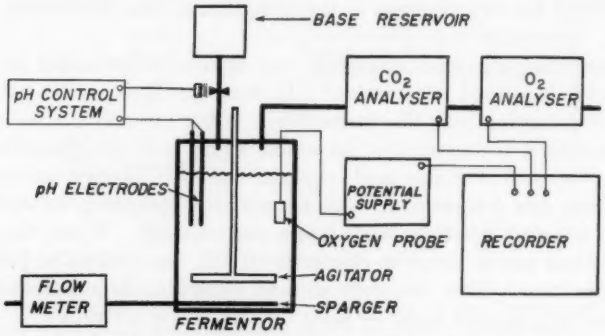

Fig. 5. Apparatus for the study of aeration in fermentations

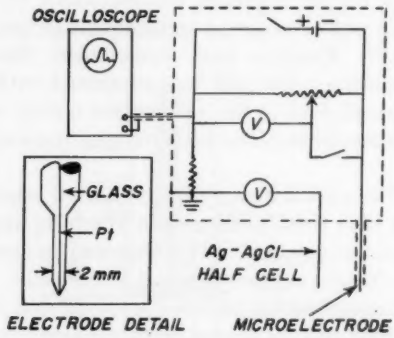

Fig. 6. Apparatus for platinum microelectrode measurement of dissolved oxygen. A piece of No. 32 platinum wire was sealed in a 2-mm pyrex tube and the end was ground to a point

(as a voltage drop across a suitable resistor) with a Hewlett–Packard Model 130A oscilloscope. The oscilloscope traces were recorded photographically. The fermentor was used as the reference half-cell because it was convenient to ground one side of the circuit for recording purposes. However, the potential of the 'fermentor half-cell' was not constant. Consequently, the potential of the 'fermentor half-cell' was periodically measured with respect to a Ag–AgCl half-cell and the applied potential was

corrected for any changes in the potential of the 'fermentor half-cell'.

The platinum microelectrode was similar in principle to previously described electrodes.[6] It was sterilized with ethanol before insertion into the fermentor. After insertion, a potential of about 0·4 V was applied for about 5 min and then the relationship between voltage and current for the oxygen-containing medium was determined. After a suitable operating voltage had been selected, readings were taken periodically. When the electrode was not in use, the electrode circuit was broken to prevent polarization. This electrode showed considerable polarization in fermentations and could be used for only a few hours.

At intervals, samples were removed from the fermentations. The sugar concentration was determined by copper reduction.[30] Dry-cell weights for the bacterial fermentations were determined turbidimetrically with occasional calibration against direct weight determinations.[20] Weights were determined directly for the mould fermentations. The pH was measured with a glass electrode and any small drift in the pH control system was corrected. The method for determination of the oxygen demand needs a little explanation.

For bacterial fermentations, suitably diluted samples of culture were incubated with fresh medium in a Warburg apparatus under conditions of excess aeration. The observed oxygen uptake rate was assumed to be the oxygen demand of the cells.

For mould fermentations, samples were diluted with fresh air-saturated medium and incubated with no aeration in the vessel shown in Fig. 7. The oxygen tension was measured with a rapidly responding probe (see Fig. 8) and automatically recorded. A typical curve for a *Penicillium chrysogenum* sample is shown in Fig. 9. As long as the oxygen tension was above the critical value, the oxygen utilization rate was constant. Consequently, if the solubility of oxygen in the medium were known, the oxygen utilization rate (oxygen demand) could be calculated.

We attempted to determine the solubility of oxygen in the medium by titration of the air-saturated medium with standardized sodium sulphite and by the Winkler method. Both methods were unsuccessful because medium components interfered with the analyses. Therefore, the following method was used. The

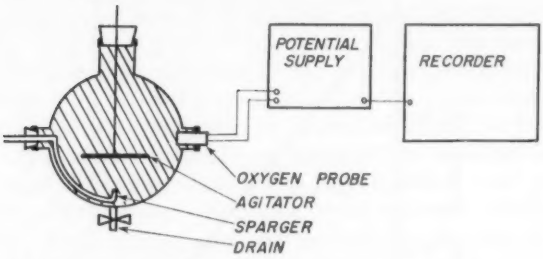

Fig. 7. Apparatus for the evaluation of true critical oxygen tension and oxygen demand. The vessel was constructed from a 1-l. flask. Temperature was automatically controlled at 30°C with a thermistor controller and an infrared lamp. Dissolved oxygen was measured with two rapidly responding oxygen probes (see Fig. 8) and automatically recorded. The sparger was used for saturation of the medium with air and for determination of the calibration curves for the probes. No gas phase was present in the vessel during an experiment

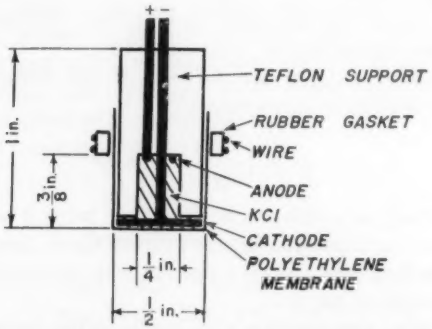

Fig. 8. Rapidly responding oxygen probe. This probe had a large KCl reservoir. Both the anode and cathode were silver. For conductivity, four 0·01-in. holes were drilled through the cathode. The calibration curve for this probe was not linear and, as a result, it had to be periodically determined. The probe reached 90 per cent response in about 4 min

medium was sparged with nitrogen to exhaust all of the dissolved oxygen and then a known quantity of pure oxygen was added to the vessel. After absorption of the oxygen, the increase in the oxygen tension of the medium was recorded. The solubility of oxygen was calculated after a correction was applied for the

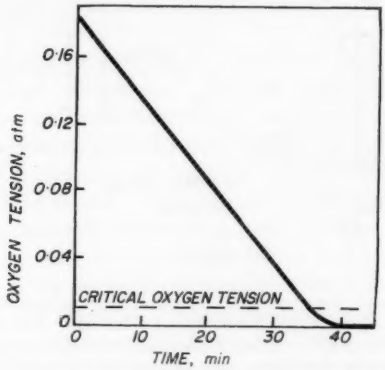

Fig. 9. Oxygen removal from solution by cells of *Penicillium chrysogenum* W49–133. The cells were grown in shake flasks and diluted with fresh, air-saturated medium

amount of oxygen in the small gas phase. We found that the solubility was 0·88 mmoles/l. atm at 30°C.

The calculated oxygen uptake rate of the fermentations was obtained by the following equation.[15]

$$N = \left(\frac{3333}{y} - 41{\cdot}8\right)G$$

where N = oxygen uptake rate, mmoles/l. min; y = yield, grams dry cells produced per 100 grams glucose used; and G = growth rate, g dry cells/l. min. This equation was derived from material balance considerations.

The following assumptions were made in the derivation.

1. Glucose is the only carbon source.
2. The cells have the same composition as yeast.
3. Cells, carbon dioxide and water are the only products of metabolism.

Fermentation Media

The medium for the *Aerobacter aerogenes* and *Escherichia coli* fermentations was KH_2PO_4, 0·7 per cent; $(NH_4)_2HPO_4$, 0·5 per cent; Na_2SO_4, 0·05 per cent; $MgSO_4{\cdot}7H_2O$, 0·08 per cent; NaCl,

0·004 per cent; $FeSO_4 \cdot 7H_2O$, 0·004 per cent; $MnSO_4 \cdot 4H_2O$, 0·004 per cent; antifoam (GE-60), 0·03 per cent; glucose, 5 per cent. Usually, additional glucose was added during the fermentation to ensure sufficient substrate. The pH was automatically controlled at $7{\cdot}0 \pm 0{\cdot}1$ with ammonia and the temperature of all fermentations was automatically controlled at $30° \pm 0{\cdot}5$°C.

For *Azotobacter vinelandii*, a nitrogen-free medium was normally used. Its composition was $MgSO_4 \cdot 7H_2O$, 0·02 per cent; K_2HPO_4, 0·09 per cent; KH_2PO_4, 0·023 per cent; $CaSO_4$, 0·001 per cent; ferric citrate and sodium molybdate, each 3 p.p.m.; antifoam (GE-60), 0.03 per cent; glucose, 5 per cent. The pH was automatically controlled at $7{\cdot}0 \pm 0{\cdot}1$ with potassium hydroxide. When these cells were grown in the presence of ammonia as a nitrogen source, 0·1 per cent ammonium sulphate was added to the above medium and the pH was controlled with ammonia.

For *Penicillium chrysogenum* and *Aspergillus niger* the following medium was used: KH_2PO_4, 0·25 per cent; $MgSO_4 \cdot 7H_2O$, 0·1 per cent; $(NH_4)_2$ citrate, 0·1 per cent; $(NH_4)_2SO_4$, 0·25 per cent; $ZnCl_2$, 0·0005 per cent; $FeSO_4 \cdot 7H_2O$, 0·003 per cent; $CuCl_2$, 0·0001 per cent; $MnSO_4 \cdot H_2O$, 0·0003 per cent; antifoam (GE-60), 0·03 per cent; glucose, 5 per cent. The pH was automatically controlled with ammonia at $4{\cdot}0 \pm 0{\cdot}1$ for *A. niger* and at $5{\cdot}0 \pm 0{\cdot}1$ for *P. chrysogenum*.

Fermentation Equipment

Two fermentors were used. The smaller had a working volume of $3{\cdot}5 \pm 0{\cdot}5$ l. It was 31 cm high and the diameter was 15 cm. Agitation was accomplished with a 4-bladed propeller 12 cm in diameter. The blades were inclined 30° from the horizontal, causing upward pumping of the liquid. In most fermentations, the agitator speed was 920 rev/min and the air flow rate was 2 l./min. The aeration efficiency as measured with the sulphite oxidation method was 1·3 mmoles O_2/l. min.

The large fermentor had a working volume of $1{,}900 \pm 70$ l. It was 274 cm high and had an inside diameter of 122 cm. It was agitated by a vaned disc impeller 66 cm in diameter, operating at 155 rev/min. The air flow rate was 850 l./min. The aeration

efficiency of this vessel as measured with the sulphite oxidation method was 2·9 mmoles O_2/l. min.

Both fermentors had perforated pipe spargers and were arranged for automatic pH control. The broth temperature in both vessels was automatically controlled at 30° ± 0·5°C. Dissolved oxygen was usually measured with two probes in the small fermentor and with six probes in the large fermentor.

Results and Discussion

Critical Oxygen Tension

Available data indicate that the metabolic rate of microorganisms is practically independent of oxygen tension if this is above the critical value (about 0·01 atm). Consequently, an aerobic fermentation should exhibit no signs of oxygen deficiency until the oxygen level becomes very low.

The effect of dissolved oxygen on the oxygen utilization rate of several cultures is shown in Fig. 10. In these experiments, the

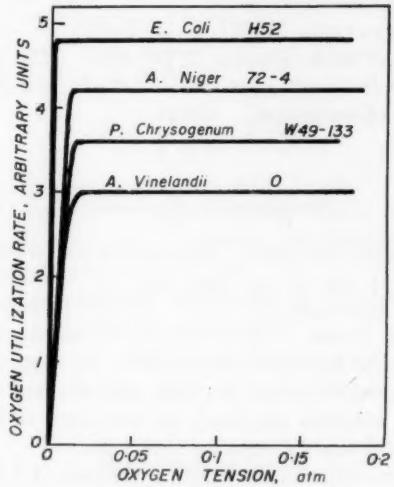

Fig. 10. Effect of dissolved oxygen on oxygen utilization rates of several cultures, diluted with fresh, air-saturated medium and incubated in the vessel shown in Fig. 7. These results were calculated from data similar to that shown in Fig. 9

cells were grown in shaken flasks, diluted with fresh, air-saturated medium and incubated with no aeration in the vessel shown in Fig. 7. The oxygen utilization rates of these cultures were independent of dissolved oxygen as long as the oxygen tension was above the critical values. The critical values agree well with available data.[12]

Aeration in Bacterial Fermentations

Data obtained on an *Aerobacter aerogenes* fermentation in the 3·5-l. fermentor are summarized in Fig. 11. It will be noted that

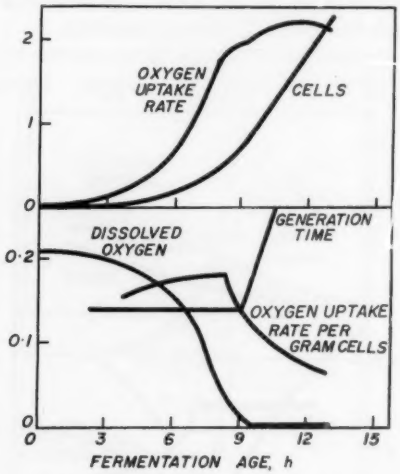

Fig. 11. *Aerobacter aerogenes* fermentation in the 3·5-l. fermentor. The air-saturated dissolved-oxygen level for this vessel was 0·21 atm. Units: cells, g/100 ml; dissolved oxygen, atm; generation time, tens of hours; oxygen uptake rate, mmoles O_2/l. min.; oxygen uptake rate per gram of cells, mmoles O_2/0·5 g cells min

as the cell weight exponentially increased, the dissolved-oxygen level fell. Oxygen deficiency, as measured by the oxygen utilization rate per gram of cells, became apparent at 8·2 h, when the dissolved-oxygen level was about 0·02 atm. Oxygen deficiency as measured by the change in cell growth rate (from exponential

to linear growth) appeared at 9 h when the dissolved-oxygen level was about 0·01 atm.

The apparent critical oxygen tension is approximately that which might be expected from the data of various workers.[12] Therefore, variations in dissolved oxygen did not appear to be important in this fermentation. Actually, we have always observed good agreement between probes at various locations and between the probe and tubing method[26] for measurement of dissolved oxygen in bacterial fermentations in this vessel. This indicates that large-scale variations in oxygen tension (due to insufficient bulk mixing) were not present. Probably small-scale variations (due to bubble trails) caused a small difference between the apparent and true critical oxygen tensions.

Data on an *Aerobacter aerogenes* fermentation in the 1,900-l. fermentor are given in Fig. 12. Oxygen deficiency became

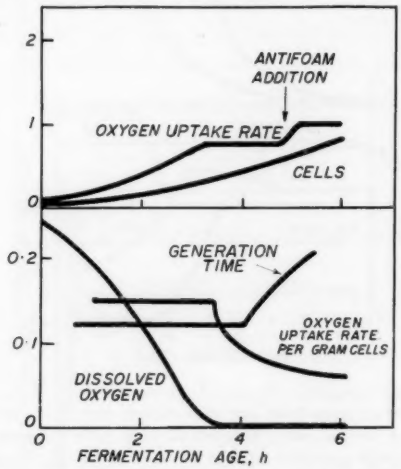

Fig. 12. *Aerobacter aerogenes* fermentation in the 1,900-l. fermentor. The air-saturated dissolved oxygen level for this vessel (because fermentor pressure was above atmospheric) was 0·28 atm. Units: cells, g/100 ml; dissolved oxygen, atm; generation time, tens of hours; oxygen uptake rate, mmoles O_2/l. min; oxygen uptake rate per gram cells, mmoles O_2/0·5 g cells min

apparent at a dissolved-oxygen tension of about 0·01 atm, as in the bacterial fermentation in the small fermentor. Because the

aeration efficiency of this fermentor was very low in the presence of cells, as will be shown later, the cell density was lower. Dissolved oxygen was measured at six positions in this fermentation. Since a good correlation between the apparent and true critical oxygen tension was observed, good agreement between the individual probes would be expected. This was the case. Therefore, variations in dissolved oxygen in the fermentations did not appear to be important. It is interesting to note that when antifoam was added to this fermentor, aeration efficiency increased.

Data on an *Escherichia coli* fermentation in the small fermentor are summarized in Fig. 13. For the first 10·8 h, the cells exhibited typical aerobic metabolism. The yield was high. The cells grew

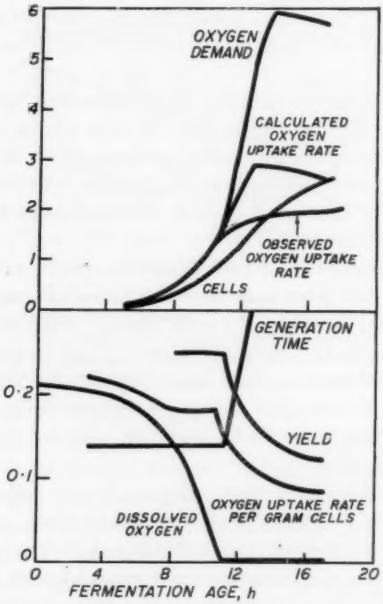

Fig. 13. *Escherichia coli* H52 fermentation in the 3·5-l. fermentor. Units: calculated oxygen uptake rate, observed oxygen uptake rate and oxygen demand, each mmoles/l. min; cells, g/100 ml; dissolved oxygen, atm; generation time, tens of hours; oxygen uptake rate per gram cells, mmoles O_2/g cells min; yield, g cells produced per 0·5 g sugar used

exponentially. The oxygen uptake rate per gram of cells was fairly constant. The calculated and observed oxygen uptake rates showed good agreement. The oxygen demand, as measured with the Warburg apparatus, agreed well with the observed oxygen uptake rate.

However, after the dissolved-oxygen tension fell below 0·01 atm, signs of oxygen deficiency were observed. The oxygen uptake rate per gram of cells showed a sharp decrease, indicating that oxygen was limiting metabolism. The oxygen demand was greater than the observed uptake rate, indicating that the demand was greater than the supply. The yield showed a sharp decrease and the calculated oxygen uptake rate exceeded the observed rate, indicating anaerobic metabolism. The cell growth rate changed from exponential to less than linear growth, indicating that oxygen deficiency was not only limiting growth, it was actually damaging the cells.

The critical oxygen tension for *Azotobacter vinelandii*, evaluated with the apparatus shown in Fig. 7, was about 0·02 atm (see Fig. 10). However, when these cells were grown in the 3·5-l. fermentor, the apparent critical oxygen tension was very difficult to evaluate. The results of a typical fermentation with nitrogen-free medium are shown in Fig. 14.

The oxygen demand and the observed uptake rate agree fairly well up to 11 h, but after this time the demand was much greater than the observed uptake rate even though the dissolved-oxygen level was far above the critical value. High oxygen demand in *Azotobacter* fermentations had been previously observed.[24] At that time it was shown that the rapid oxygen utilization of dilute suspensions in Warburg flasks was not due to the use of fresh medium in the flasks.

It might be concluded that after 11 h the cells were oxygen starved, since they immediately increased their oxygen uptake rate upon being placed under conditions of excess oxygen supply. However, the oxygen uptake rate per gram of cells was fairly constant until 14 h. The generation time was minimal from 13 to 20 h and the yield was maximal only after 21 h. Consequently, it appeared that in the presence of excess oxygen, the *Azotobacter* cells wasted oxygen, burning sugar at a rate greater than necessary to supply their energy requirements.

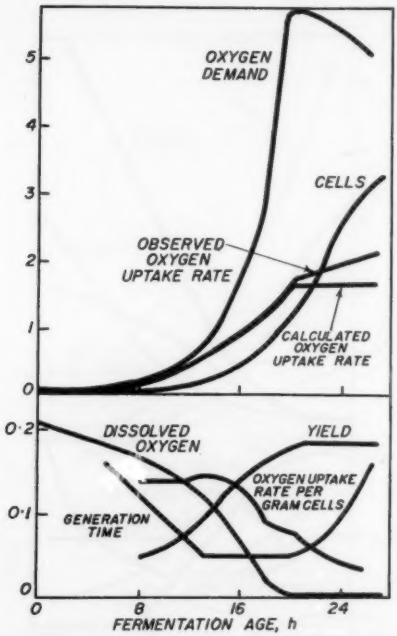

Fig. 14. *Azotobacter vinelandii* O fermentation in the 3·5-l. fermentor. The cells were grown on a nitrogen-free medium. Units: calculated oxygen uptake rate, observed oxygen uptake rate and oxygen demand, each mmoles/l. min; cells, g/500 ml; dissolved oxygen, atm; generation time, h × 50; oxygen uptake rate per gram cells, mmoles O_2/0·1 g cells min; yield, g cells produced per g sugar used

It appears reasonable that a very low oxidation potential is necessary for the nitrogen fixation reactions in *Azotobacter* cells. If this is true, the 'oxygen wasting system' might play an important role in the reduction of the intracellular oxidation potential.

To investigate this peculiar metabolism further, *Azotobacter* cells were grown in the presence of an ammonia nitrogen source. The results of the fermentation are summarized in Fig. 15. It is obvious that the 'oxygen wasting system' was operative in the presence of an ammonia nitrogen source.

The 'oxygen wasting system' appeared to be affected by the dissolved-oxygen level because the cells immediately increased

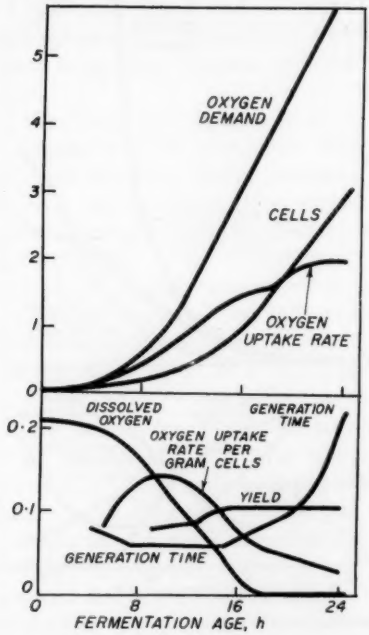

Fig. 15. *Azotobacter vinelandii* O fermentation in the 3·5-l. fermentor. The cells were grown in the presence of an ammonia nitrogen source. Units: cells, g/500 ml; dissolved oxygen, atm; generation time, h × 50; oxygen demand and oxygen uptake rates, each mmoles O_2/l. min; oxygen uptake rate per gram cells, mmoles O_2/0·1 g cells min; yield, g cells produced per g sugar used

their oxygen uptake rate upon being placed under conditions of excess oxygen. Therefore, we attempted to increase the total cell yield by manual control of dissolved oxygen. The results of this fermentation with a nitrogen-free medium are shown in Fig. 16. It is apparent that the low dissolved-oxygen level in this fermentation inhibited the 'oxygen wasting system' because the oxygen demand was much greater than the observed oxygen uptake rate and the oxygen uptake rate per gram of cells was relatively low throughout the fermentation. Apparently the 'oxygen wasting

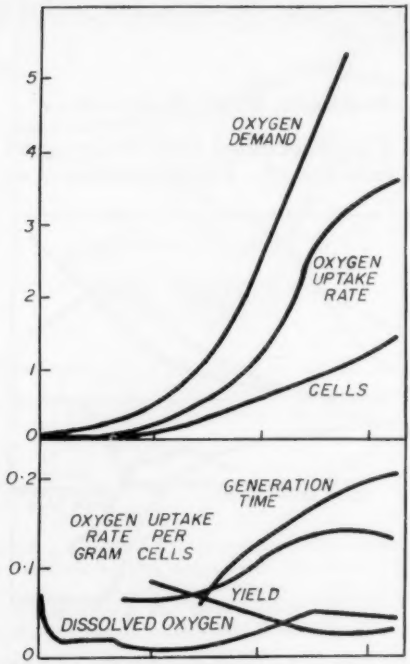

Fig. 16. *Azotobacter vinelandii* O fermentation in the 3.5-l. fermentor. The cells were grown on a nitrogen-free medium and the dissolved-oxygen level was manually controlled. The following agitation and air flow rates were used.

Time, h	Agitation, rev/min	Air flow, l./min	Time, h	Agitation, rev/min	Air flow, l./min
0–1	200	0·16	13–14	640	1·38
1–2	280	0·16	14–15	760	1·99
2–3	330	0·16	15–16	840	1·99
3–6	400	0·16	16–18	890	1·99
6–8	480	0·16	18–19	890	2·72
8–9	520	0·16	19–20	1200	3·29
9–11	520	0·78	20–21	1290	3·29
11–12	580	0·78	21–22	1560	3·29
12–13	590	1·38	22–28	1750	3·29

Units: cells, g/500 ml; dissolved oxygen, atm; generation time, h × 50; oxygen demand and oxygen uptake rates, mmoles O_2/l. min; oxygen uptake rate per g of cells, mmoles O_2/0·1 g cells min; yield, g cells produced per g sugar used

system' is not solely responsible for the low yield in the *Azotobacter* fermentations.

Aeration in Mould Fermentations

The progress of an *Aspergillus niger* fermentation in the 3·5-l. fermentor is shown in Fig. 17. Oxygen deficiency, as measured by

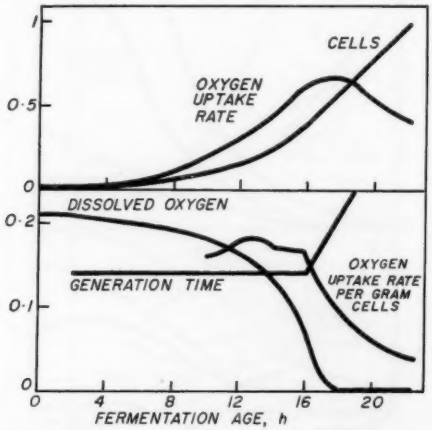

Fig. 17. *Aspergillus niger* H72-4 fermentation in the 3·5-l. fermentor. Units: cells, g/50 ml; dissolved oxygen, atm; generation time, h × 20; oxygen uptake rate, mmoles O_2/l. min; oxygen uptake rate per gram cells, mmoles O_2/2 g cells min

the beginning of linear growth or by the oxygen uptake rate per gram of cells, began at 16 h when the measured dissolved-oxygen tension was 0·07 atm. Dissolved oxygen was measured with two probes, located at the same level, and no difference was observed.

When these cells were grown in the 1,900-l. fermentor, the data shown in Fig. 18 were obtained. During the period from 12 to 14 h, growth changed from exponential to linear and the oxygen utilization rate per gram of cells began to decrease. The dissolved-oxygen level during this period was high, 0·12 to 0·15 atm. Dissolved oxygen was measured with six probes and no significant differences were observed.

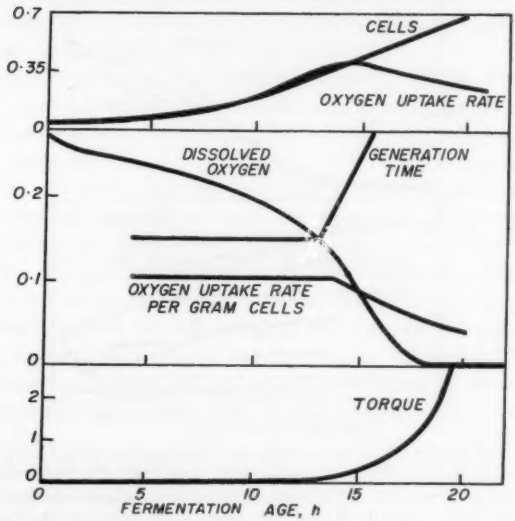

Fig. 18. *Aspergillus niger* H72-4 fermentation in the 1,900-l. fermentor. Units: cells, g/50 ml; dissolved oxygen, atm; generation time, h × 20; oxygen uptake rate, mmoles O_2/l. min.; oxygen uptake rate per gram cells, mmoles O_2/2 g cells min; torque, arbitrary units. The torque is that required to initiate rotation of a small turbine impeller immersed in a sample of culture liquid removed from the fermentor

Samples were removed from the above fermentation and the torque required to initiate rotation of a small turbine impeller in the culture was recorded. When the cell concentration was low, the fluid exhibited Newtonian-viscosity behaviour and practically no torque was required to start rotation of the impeller. However, when the population was high, the culture behaved as a Bingham plastic[10] and considerable torque was required to initiate rotation. It is interesting to note that signs of oxygen deficiency occurred shortly after the culture began to exhibit non-Newtonian-viscosity behaviour.

Data on a *Penicillium chrysogenum* fermentation in the 3·5-l. fermentor are shown in Fig. 19. In this fermentation, the cells were not able to decrease the dissolved-oxygen level to zero. The oxygen demand, as measured with the apparatus shown in Fig. 7,

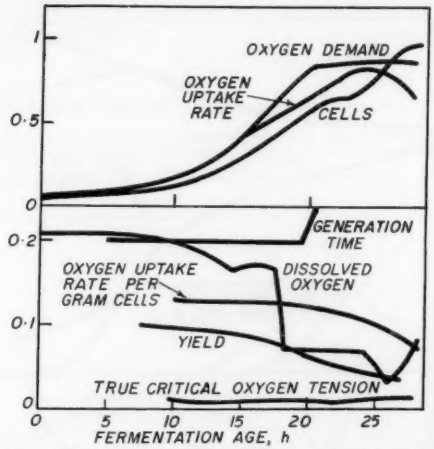

Fig. 19. *Penicillium chrysogenum* W49-133 fermentation in the 3·5-l. fermentor. Units: cells, g/50 ml; dissolved-oxygen and true critical oxygen tension, each atm; generation time, h × 20; oxygen demand and oxygen uptake rates, each mmoles O_2/l. min; oxygen uptake per gram cells, mmoles O_2/2 g cells min; yield, g cells produced per 0·2 g sugar used

agreed well with the observed uptake rate until 17 h. At 17·8 h, 1 ml of antifoam (GE-60) was added to the fermentation to suppress foaming. This caused a large drop in the dissolved-oxygen level. However, the recorded dissolved-oxygen level was never below the measured critical level.

Evaluation of the apparent critical oxygen tension for this fermentation is very difficult. However, on the basis of the generation time and the relation between the oxygen demand and uptake rate, one might conclude that the apparent value is between 0·15 and 0.08 atm.

One of the most obvious differences between bacterial and mould fermentations is the correlation between the apparent and true critical oxygen tensions. The poor correlation in mould fermentations was probably due to variations in dissolved oxygen, but the nature of these variations was not obvious. It seemed unlikely that bubble trails accounted for these variations because they had practically no effect in the bacterial fermentations. Large-scale variations, due to poor bulk mixing, seemed unlikely

because fairly good agreement among the individual probes was always observed. Consequently, it appeared that variations due to mycelial clumps[2] might be important.

Mycelial clumps may occur in two forms. First, rigid aggregates of tightly packed mycelium (pellets) may be present in a viscous fermentation. These are often observed under conditions of small inoculum or low agitation. Secondly, because mould suspensions exhibit non-Newtonian-viscosity behaviour, semi-rigid clumps of mycelium may be present. When gradually increasing shear is applied to a mould suspension, it behaves as a rigid body. When the shear reaches a certain value, the suspension fractures more or less in a plane, giving two rigid regions moving with respect to each other. The higher the value of the shear, the more closely spaced are the fracture planes. Therefore, mycelial suspensions under agitation may consist of semi-rigid clumps of mycelium, any single clump having only a transitory existence.

In an attempt to estimate the effect of clump size on apparent critical oxygen tension, the following assumptions were made:

1. The clumps are spherical.
2. Oxygen deficiency occurs when the oxygen tension at the centre of the clump is less than the true critical oxygen tension.
3. The diffusivity of oxygen in the clump is equal to the diffusivity of oxygen in water times the water content of the clump.
4. The solubility of oxygen in the clump is equal to the solubility of oxygen in the medium.

With these assumptions, it is possible to derive the following equation:

$$R = \{12DS[(pO_2)_L - (pO_2)_c]/Q\}^{1/2}$$

where D = diffusivity of oxygen in the clump, cm^2/min; $(pO_2)_c$ = true critical oxygen tension, atm; $(pO_2)_L$ = apparent critical oxygen tension, atm; Q = oxygen utilization rate of cells in clump, mmoles O_2/l. min; R = clump radius necessary to produce discrepancy between $(pO_2)_c$ and $(pO_2)_L$, cm; and S = solubility of oxygen in the clump, mmoles O_2/l. atm.

In the fermentation summarized in Fig. 19, the apparent critical oxygen tension, $(pO_2)_L$, was 0·08 atm. The true critical oxygen tension, $(pO_2)_c$, was about 0·01 atm. The solubility of oxygen

was 0·88 mmoles/l. atm, and the oxygen uptake rate of the cells was 0·06 mmoles/g min. If rigid pellets were present and the pellets contained no extracellular water, the pellets would contain about 70 per cent intracellular water. For this case, $D = (1{\cdot}41 \times 10^{-3}$ cm^2/min)(0·7) and $Q = (0{\cdot}06$ mmoles O_2/g min) (300 g/l.). Therefore, the clump diameter would have to be 0·12 mm. It seems unlikely that pellets were present in this fermentation because the true critical oxygen tension, evaluated with cells from the fermentor, was about 0·01 atm throughout the fermentation. If semi-rigid clumps were present, $D = (1.41 \times 10^{-3}$ cm^2/min) (0·92) and $Q = 0{\cdot}55$ mmoles O_2/l. min. Therefore, the semi-rigid clump diameter would have to be 0·83 mm.

On the basis of these calculations, it seemed possible that semi-rigid mycelial clumps could account for the poor correlation between the apparent and true critical oxygen tension in the mould fermentations. Therefore, a *Penicillium* fermentation was conducted with platinum-microelectrode measurements of dissolved oxygen in an attempt to determine the importance of mycelial clumps. The results are shown in Fig. 20.

The two probes and the microelectrode were placed at the same level in the fermentor, approximately half way between the

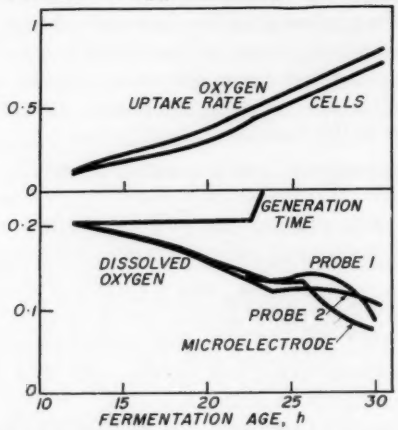

Fig. 20. *Penicillium chrysogenum* W49-133 in the 3·5-l. fermentor. Units: cells, g/50 ml; dissolved oxygen, atm; generation time, h × 20; oxygen uptake rate, mmoles O_2/l. min

bottom and the liquid surface. As the fermentation progressed, it was obvious that the microelectrode was operating successfully because occasional bubble trails were observed, but there was no evidence of low dissolved oxygen due to mycelial clumps. Between 23 and 30 h, considerable variation in dissolved oxygen was observed, but there was no evidence that the oxygen tension was below the true critical value. However, just before the sugar was exhausted, the microelectrode was placed in a different location and it indicated essentially no dissolved oxygen in this region. Consequently, it appeared that insufficient bulk mixing caused large variations in dissolved oxygen which caused the poor correlation between the apparent and true critical oxygen tension.

After the sugar was exhausted from the fermentation shown in Fig. 20, the probes and microelectrode were recalibrated and then more sugar was added to the medium. Some autolysis of the mycelium occurred during recalibration and, as a result, the viscosity of the suspension decreased. The effect of agitation of this suspension on the dissolved-oxygen tension at various locations

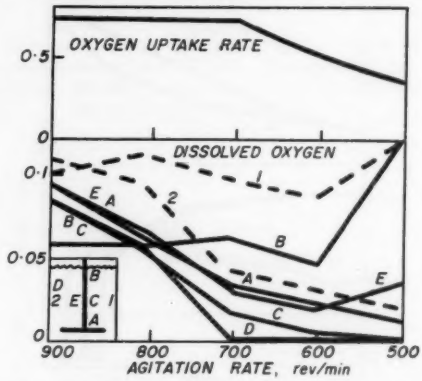

Fig. 21. Effect of agitation on dissolved oxygen and oxygen uptake rate in a *Penicillium* culture. This experiment was performed with the cells from the fermentation summarized in Fig. 20. Dissolved oxygen was measured with two oxygen probes (1 and 2), and the platinum microelectrode located at positions A, B, C, D and E. The probes were about 3 cm from the fermentor wall. Positions A, B, D, and E were about 2 cm from the wall and position C was about 1 cm from the agitator shaft. The relative positions where dissolved oxygen was measured are shown in the lower left corner of the figure. Units: dissolved oxygen, atm; oxygen uptake rate, mmoles O_2/l. min

in the 3·5-l. fermentor and on the oxygen uptake rate is shown in Fig. 21. Sufficient oxygen was available above an agitation rate of 700 rev/min because the oxygen uptake rate was independent of agitation. However, below 700 rev/min, the oxygen uptake rate was dependent on the agitation rate and the dissolved-oxygen level in some locations was very low, indicating that oxygen was limiting in some areas of the fermentor. Actually, one might conclude that at 500 rev/min, about half of the cells were devoid of oxygen because the oxygen uptake rate was only half of the maximum rate. The tremendous variation in dissolved oxygen was probably due to non-uniform bubble distribution.

If it were possible to achieve good mixing in a *Penicillium* fermentation, a good correlation between the apparent and true critical oxygen tension might be expected. In an attempt to increase mixing, the volume of the medium for a *Penicillium* fermentation was decreased to 2 l. The results are shown in Fig. 22. It is apparent that after 25·5 h, large variations in dis-

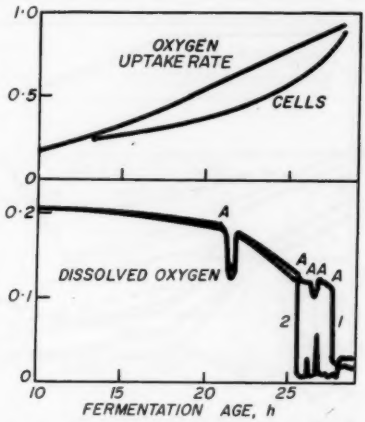

Fig. 22. *Penicillium chrysogenum* W49-133 fermentation in the 3·5-l. fermentor. Dissolved oxygen was measured with two probes. Their locations are shown in the lower left corner of Fig. 21. Antifoam additions are denoted by A. Units: cells, g/50 ml; dissolved oxygen, atm; oxygen uptake rate, mmoles O_2/l. min

solved oxygen were present, indicating that mixing was still insufficient. It is interesting to note the effect of antifoam addi-

tions on the dissolved-oxygen level. Antifoam usually decreased bubble hold-up and as a result the dissolved-oxygen level decreased. However, when mixing was not sufficient, antifoam appeared to enhance mixing and, as a result, the dissolved-oxygen level in the region of poor mixing usually increased.

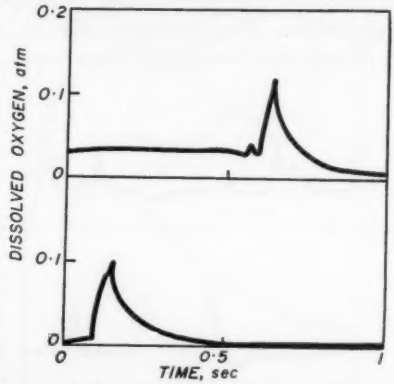

Fig. 23. Short-time variations in dissolved oxygen. These data were obtained from the experiment summarized in Fig. 21. The agitation rate was 503 rev/min and the microelectrode was at position E for the upper curve and at position C for the lower curve (see Fig. 21)

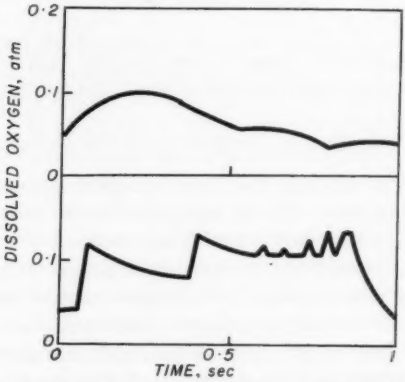

Fig. 24. Short-time variations in dissolved oxygen. These data were obtained from the experiment summarized in Fig. 21. The agitation rate was 810 rev/min and the microelectrode was at position D for both curves (see Fig. 21)

Some of the short-time variations that have been observed in *Penicillium* fermentations are shown in Figs. 23, 24 and 25. The data shown in Figs. 23 and 24 were obtained from the experiment summarized in Fig. 21. The variations shown in Fig. 23 were probably due to bubble trails. In Fig. 24, the variations were slower and were probably due to volumes of oxygen-rich liquid passing near the electrode. Data shown in Fig. 25 were obtained

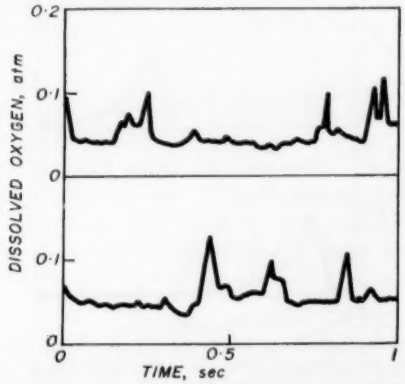

Fig. 25. Short-time variations in dissolved oxygen. These data were obtained from the fermentation summarized in Fig. 22 at 28 h. The microelectrode was at position A (see Fig. 21)

from the fermentation summarized in Fig. 22. Many bubble trails were observed near the impeller at this time.

Evaluation of Aeration in Fermentations

Sulphite oxidation and the polarographic gassing-out method permit determination of the oxygen-transfer capabilities of a fermentor. When properly used, they measure the rate at which oxygen is transferred from the gas phase to the bulk of the liquid. However, they can measure the oxygen-transfer rate only under the conditions of viscosity, surface tension and other variables prevailing at the time of the test. When both dissolved oxygen and oxygen utilization rates are determined during an actual fermentation, the oxygen-transfer capabilities of the fermentor at any time during the fermentation may be calculated. Since the driv-

ing force is the difference in oxygen tension between the bubbles and the solution, the calculation is simple.

In Fig. 26, the maximum aeration efficiency of the 3·5-l. fermentor is plotted as a function of cell concentration. The data

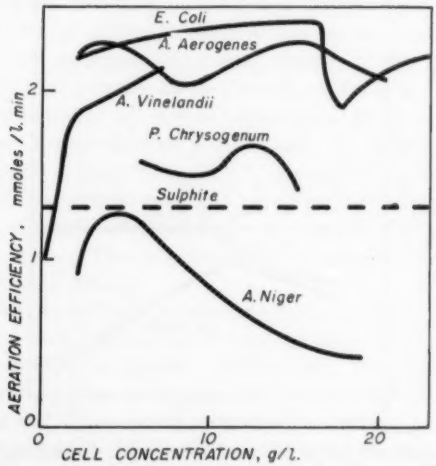

Fig. 26. Oxygen-transfer capability of the 3·5-l. fermentor. The aeration efficiency was calculated from dissolved-oxygen and oxygen-uptake-rate data

were taken from the fermentations discussed above and from some others. It will be noted that the presence of bacteria raised the oxygen-transfer coefficient to a value much above that obtained with sulphite. It was observed through the glass walls of the fermentor that the bubble size decreased as the population increased. This would be expected to increase oxygen transfer. In the mould fermentations, the bubble size decreased as the population increased but when the cultures began to exhibit non-Newtonian-viscosity behaviour, the bubble size increased. Therefore, the absorption coefficient increased at first and then decreased. When antifoam was added, the bubble size increased and, as a result, the absorption coefficient usually decreased.

In Fig. 27, the aeration efficiency of the 1,900-l. fermentor is plotted as a function of cell concentration. Comparison of Figs. 26 and 27 shows that although the large fermentor gave about

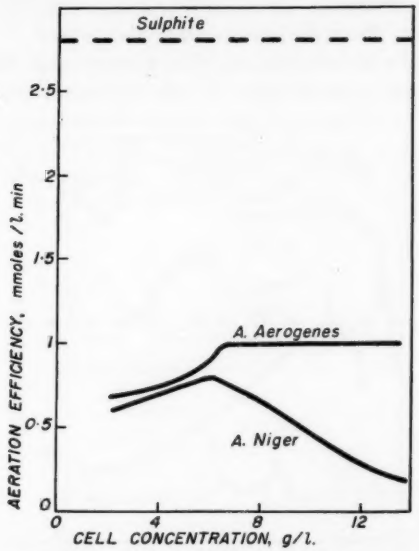

Fig. 27. Oxygen-transfer capability of the 1,900-l. fermentor. The aeration efficiency was calculated from dissolved-oxygen and oxygen-uptake-rate data. Antifoam was added only once (at 5 h) to the *A. aerogenes* fermentation. It was necessary to use automatic foam control for the *A. niger* fermentation

twice the oxygen-transfer rate of the small fermentor when evaluated with sulphite, in the presence of either bacteria or mould its performance was much poorer than that of the small fermentor. It is interesting to note that antifoam addition to the large fermentor increased the absorption coefficient. Power data on the large fermentor indicated that air flooding of the impeller was occurring in fermentations.

Conclusions

The metabolic rate of micro-organisms is not affected by dissolved oxygen until oxygen tension falls to a very low level (about 0·01 atm). Consequently, aeration is sufficient whenever the dissolved oxygen at every point in the fermentation is above the critical level. Measurement of dissolved oxygen indicates the

adequacy of aeration in fermentations, but when aeration is not sufficient, these measurements provide no indication of the magnitude of the deficit. The most useful data for evaluation of aeration are the oxygen demand rate, oxygen uptake rate and the dissolved-oxygen tension. From the latter two, the oxygen-transfer capability of the fermentor can be calculated, and when aeration is not adequate, the magnitude of the deficit can be calculated from the demand- and uptake-rate data.

Several symptoms of oxygen deficiency have been observed. When oxygen supply limited metabolism, the oxygen uptake rate per gram of cells usually decreased. Anaerobic metabolism, under conditions of inadequate aeration, often caused a decrease in yield and a poor correlation between the calculated and observed oxygen-uptake rates. The generation time usually increased when aeration became limiting.

Oxygen deficiency had various effects on the fermentations. In the *Aerobacter* and *Aspergillus* fermentations, cell-growth rate was linear when aeration limited metabolism. Therefore, the deficiency did not appear to damage the cells. On the other hand, symptoms of cell damage were observed when oxygen supply was limiting in the *Escherichia* and *Penicillium* fermentations. In the former, the cell-growth rate was less than linear after aeration was limiting. In a *Penicillium* fermentation, the oxygen demand was constant during the latter part of the fermentation, indicating that no net increase in metabolic activity occurred even though the cell concentration increased. In the *Azotobacter* fermentations, aeration had a marked effect on metabolism but interpretation of the data on these fermentations is difficult.

In bacterial fermentations, a good correlation between the apparent and true critical oxygen tensions was observed. Symptoms of oxygen deficiency were observed when the dissolved-oxygen level fell to 0·01–0·02 atm. Therefore, variations in dissolved oxygen did not appear to be important. Direct evaluation of absorption coefficients under fermentation conditions was possible. However, in mould fermentations, apparent critical oxygen tensions varied from 0·07 to 0·15 atm and large variations in dissolved oxygen, due to insufficient bulk mixing, were observed. We found no evidence of variations due to mycelial clumps.

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The Chemical Sterilization of Liquid Media with Beta-Propiolactone and Ethylene Oxide *

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Summary The effectiveness of beta-propiolactone (BPL) and ethylene oxides as sterilants for liquid microbiological media was studied. Preliminary tests against heavy suspensions of *B. coagulans* in various media confirmed the sterilizing levels found by earlier investigators. In actinomycin fermentation tests, both compounds were found to sterilize effectively at concentrations up to 0·5 per cent without significant effects on the growth-promoting qualities of the medium. At the concentrations required to sterilize large numbers of spores (0·5–1·0 per cent), however, both agents exhibit inhibitory effects on the fermentation. In these cases BPL is less damaging than ethylene oxide.

Introduction

Liquid media for the growth of micro-organisms in pure culture are most frequently sterilized by autoclaving or filtration prior to use. The problems commonly encountered with these methods are substantial. Autoclaving often results in the loss of nutrient values by overheating and undesirable physical changes, such as increased colour. In filtration, nutrients may be adsorbed by the filtering medium, viruses and some bacterial forms may pass through the filter, or the filter may plug. In any case, very long time periods are required, especially when volumes of media above laboratory needs are processed.

An effective chemical sterilant for microbiological media would eliminate the disadvantages of filtration and offer advantages over heat in many cases. The main criteria for an ideal chemical sterilant are that:

(1) It is completely biocidal at low concentrations.
(2) It is innocuous to the nutrients in the media.

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(3) The residual compound is easily removed or the resulting decomposition products are inert to the growth of desired organisms.

(4) It is effective under ordinary atmospheric conditions.

(5) It is rapid in action, easy to use, inexpensive and commercially available.

(6) It is non-flammable, non-explosive, and non-toxic.

Two candidates proposed as chemical sterilants for liquid media are beta-propiolactone (BPL) and ethylene oxide.

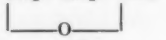
Beta-propiolactone is a colourless liquid with the formula and physical properties listed in Table I.[1] It is commercially available at 96 per cent purity or specially purified for biological use to 99 per cent. The chemical reactions of BPL are characteristic of the 4-membered-ring structures that are very easily opened by a wide variety of chemical reagents.[2, 3] BPL reacts readily with hydroxyl, amino, carboxyl, sulphhydryl, and phenolic groups, all of which are associated with proteins.[4] BPL has a high degree of biocidal activity; it is a broadly effective sterilant with a short life expectancy. The process of inactivation occurs during the short hydrolysis phase, and the hydrolysis product, beta-hydroxypropionic acid, has no biological activity of its own.[1]

LoGrippo, Hartman and their co-workers have reported on the effectiveness of liquid BPL against a variety of bacteria, fungi, and viruses.[5, 6, 7] In their studies, pathogenic fungi responded to 0·25–0·5 per cent BPL, vegetative bacteria required 0·1–0·2 per cent BPL, and suspensions of desiccated spores required 0·5–0·75 per cent BPL. Two-hour contact times were employed at 37°C. In these and other studies,[8] BPL was not degrading to plasma or serum proteins, as detected by electrophoretic examination. The use of BPL vapour as a space disinfectant has been reported in a recent series of papers.[9–11]

Ethylene oxide is a colourless gas at ordinary temperatures and pressures. Below the boiling point (11°C at atmospheric pressure) it is a colourless free-flowing liquid. The physical properties of ethylene oxide are also summarized in Table I.[12]

Ethylene oxide in the gaseous state has been shown by a number of investigators to be highly bactericidal, fungicidal, and virucidal.[13, 14, 15] Wilson and Bruno[16] first reported the use of

Table I. Physical properties of beta-propiolactone and ethylene oxide

	Beta-propiolactone	Ethylene oxide
Formula		
Molecular weight	72·06	44·05
Boiling point	155°C	10·7°C
Melting point	−33·4°C	−112°C
Refractive index	n_D^{20} 1·4131	n_D^4 1·3614
Density (d_4^{20})	1·149	0·891
Flash point, open cup	74°C	below −10°C
Fire point, open cup	74°C	below −10°C
Solubility	Soluble in most organic solvents; 37% by volume in water at 25°C	Complete in most organic solvents and water

liquid ethylene oxide for the sterilization of bacteriological media. Routine sterilization required 0·5–1 per cent ethylene oxide in the fluid for 1 h at ice-bath temperature.

Ginsberg and Wilson[17] inactivated several viruses with liquid ethylene oxide, including influenza, Newcastle, as well as Columbia MM and Theiler's FA mouse encephalomyelitis viruses. A concentration of 1 volume per cent was used in the presence of 90–99 per cent normal horse serum without any gross alterations in the serum. Judge and Pelczar[18] reported on the use of liquid ethylene oxide for sterilizing carbohydrate solutions prior to their use in microbiological fermentation tests. No damaging effect on the carbohydrates was detected by qualitative microbiological tests or chemical analysis. The ethylene oxide was vapourized and removed from the media by gentle heating. As indicated in Table I, ethylene oxide is vigorously flammable and the vapours form explosive mixtures with air in all proportions from 3 to 100 per cent by volume.[12] The explosion hazard is eliminated, however, by mixing the agent with an inert carrier such as carbon dioxide or the halogenated hydrocarbons. The ethylene oxide–Freon mixture sold as Cryoxide* was used in our studies. It contains 11 per cent ethylene oxide by volume and boils at 11°C.

* American Sterilizer Company, Erie, Pennsylvania.

This paper concerns itself with two aspects of the study of BPL and ethylene oxide as chemical sterilants for microbiological media:

First, a comparison of the sterilizing efficiencies of the two compounds against the heat-resistant spores of *Bacillus coagulans* suspended in various microbiological media, and secondly, a quantitative determination of the effects of chemically-treated media on a typical antibiotic fermentation system.

Experimental

The first experiments provide a comparison of the sterilizing efficiencies of these two compounds against the same test organisms. The heat resistant spores of *B. coagulans* (National Canners Association Culture 4167) were selected as the test organisms and three different biological media were used as suspending fluids. The test media included:

(1) nutrient broth, a growth medium for many common bacteria;

(2) brain–heart infusion broth plus 1 per cent glucose, a growth medium for fungi and fastidious organisms;

(3) Eagle's medium plus 10 per cent human serum, a growth medium for cells in tissue culture.[19]

Sterile tubes of test media were inoculated with known high concentrations of spores ranging from 10^4 to 10^7 spores per ml. The tubes were treated with varying concentrations of the two sterilizing compounds ranging from 0·1–1 per cent. With BPL, the contact was at room temperature (22°C); for the ethylene-oxide-treated tubes the Cryoxide addition and contact period were at refrigerator temperature (4°C). After the appropriate contact time, the tubes containing Cryoxide were held in a 55°C water bath to completely vapourize the agent. Finally, all tubes were sampled and subcultured into *B. coagulans* broth* for demonstration of viability. The results for BPL are summarized in Table II.

As indicated, BPL at 0·5–1 per cent was an effective sterilizing agent against high spore concentrations of *B. coagulans*. These

* *B. coagulans* broth (20): yeast extract 5·0 g/l., proteose peptone 5·0 g/l., glucose 5·0 g/l., K_2HPO_4 4·0 g/l.

Table II. Beta-propiolactone treatment of various media containing *B. coagulans*

Suspending medium	BPL concentration, %				
	1·0	0·5	0·2	0·1	None
Nutrient broth	−	−	+	+	+
Brain–heart infusion + 1% glucose	−	−	−	+	+
Eagle's medium + 10% serum	−	−	+	+	+

(+) Positive growth in subculture test.
(−) No growth in subculture test.
Temperature 22°C. Contact time 2 h.
Spore concentration 2×10^5/ml.

results are in good agreement with the studies of LoGrippo[5] where 0·5–0·75 per cent BPL sterilized heavy suspensions of *B. globigii* in 2 h at 37°C.

Table III presents a similar summary for the ethylene oxide experiments. As with BPL, ethylene oxide was effective at

Table III. Ethylene oxide treatment of various media containing *B. coagulans*

Suspending medium	Ethylene oxide concentration, %				
	1·0	0·5	0·2	0·1	None
Nutrient broth	−	−	+	+	+
Brain–heart infusion + 1% glucose	−	−	+	+	+
Eagle's medium + 10% serum	−	−	+	+	+

(+) Positive growth in subculture test.
(−) No growth in subculture test.
Temperature 4°C. Contact time 1 h.
Spore concentration 2×10^5/ml.

0·5–1 per cent against the spores of *B. coagulans*, a confirmation of the recommended doses of Wilson and Bruno[16] and Judge and Pelczar.[18]

After demonstrating the equal sterilizing ability of these two compounds against a hardy and tough test organism, a series of

experiments were carried out to determine quantitatively whether or not these agents had any practical future as chemical sterilants for liquid media. For this purpose the fermentation of the antibiotic actinomycin was selected as the test system.

Pfizer Culture A12882, an unclassified actinomycin-producing *Streptomyces* culture, was used as the growth organism for the fermentation studies. A composite fermentation medium containing peptone and other natural complex nutrients, glucose, and various minerals and salts was used for the growth of the test culture. All fermentations were run in cotton-stoppered, 1-l. Erlenmeyer flasks containing 250 ml of growth medium.

For the seed culture, a portion of an agar slant containing a heavy spore growth of A12882 was inoculated into a 1-l. Erlenmeyer flask containing 250 ml of autoclaved growth medium at pH 7·4. The flask was incubated at 28°C on a rotary shaker at 260 rev/min for 48 h, at which time a heavy pellet-like mycelial growth was visible and the medium was a deep-gold colour typical of actinomycin broths.

The first fermentation experiments were made to determine the effects of BPL treatment on the actinomycin fermentation. A series of 1-l. flasks containing growth medium were sterilized by autoclaving and then treated with BPL. The agent was tested at 5 levels ranging from 0·12 to 2 per cent. An untreated flask was the control culture. Following a 2-h stirring period at 22°C and a 24-h incubation at 37°C, the flasks were adjusted to pH 7·4 with sterile 20 per cent NaOH to neutralize the acid hydrolysis products. Each flask was then inoculated with 15 ml of seed culture and incubated on the rotary shaker at 28°C.

A 10-ml aliquot was removed daily from each flask and frozen. At the end of the run the samples were analyzed and the following information recorded:

(1) pH of the whole broth;

(2) mycelium dry weight;

(3) reducing sugar determined by the procedure of Folin and Malmros[21];

(4) actinomycin potency determined by the paper-disc method of bioassay using *B. subtilis* as the test organism.[22] (The assay method involved the measurement of antibiotic zones produced

by a filter-paper disc (13 mm) saturated with sample, placed on agar plates of *B. subtilis* and incubated 18–24 h at 37°C. To place all bioassay results on an actinomycin weight basis, crystalline actinomycin recovered by a solvent-extraction procedure was assayed on a weight basis by the paper-disc method. A standard curve of zone diameter as a function of actinomycin concentration was drawn up for each experiment and used to calculate the weight yield of actinomycin in the test flasks. A Fisher–Lilly zone reader was used for measurement of the antibiotic zones.)

It was observed that BPL treatment of the medium was well tolerated up to the 0·5 per cent level. Fig. 1 presents the fermentation pattern for a typical experiment in this series. At 0·5 per cent BPL and higher concentrations, there was significant effect

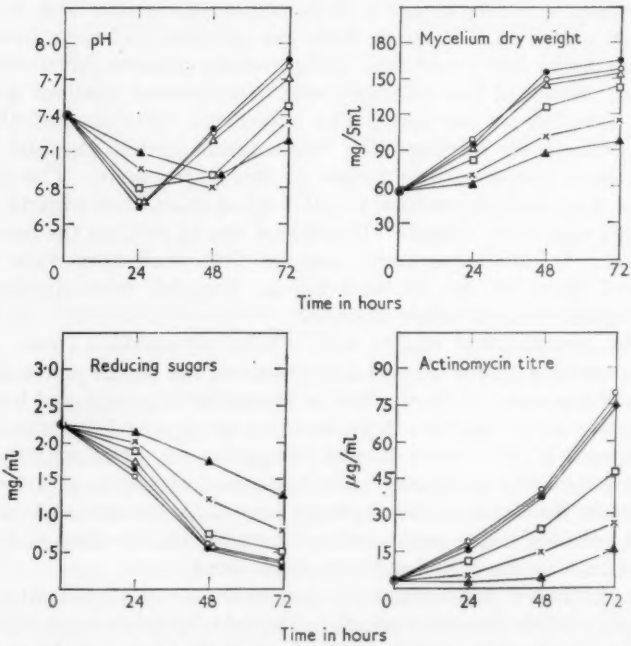


Fig. 1. Effect of beta-propiolactone treatment on actinomycin fermentation. Medium autoclaved before treatment. BPL %: ● none, ○ 0.12, △ 0.25, □ 0.50, × 1.0, ▲ 2.0

on pH pattern, sugar utilization, growth, and actinomycin titre. Considering that 0·5 per cent BPL was shown to effectively sterilize high concentrations of *B. coagulans* spores, the use of 0·2–0·5 per cent BPL in a practical test to sterilize fermentation medium without prior autoclaving was suggested.

In the next experiments, a series of flasks containing 250 ml of medium was treated with BPL without prior autoclaving. The agent was tested at three levels, 1 per cent, 0·5 per cent, and 0·25 per cent, with two flasks treated for each concentration. Immediately after BPL addition, the contents of each flask were transferred to an empty sterile flask using an aseptic technique. This was done to prevent back contamination from the unsterile cotton plug and flask surfaces of the original make-up flask. Two flasks of growth medium sterilized by autoclaving were the control cultures. Following 2 h of stirring at 22°C and a 24-h incubation period at 37°C, each flask was sampled and subcultured into nutrient broth and fluid thioglycollate medium for sterility tests. Flasks of the untreated and unautoclaved medium were also sampled for sterility. The subculture tests showed that BPL effectively sterilized the fermentation growth medium at the three concentrations tested in this experiment. The test flasks were then neutralized to pH 7·4 and inoculated with 15 ml of seed culture of A12882. Incubation was at 28°C on the rotary shaker. In this experiment, samples from each flask were removed after 24, 48, 72, and 120 h. Samples from duplicate flasks were pooled before analysis.

The fermentation results were similar to previous runs. As shown in Fig. 2, for a typical experiment, the higher concentration of 1 per cent BPL resulted in less mycelial growth and lower actinomycin production. A peak of only 50 μg per ml was attained compared to the control titre of 108 μg per ml. BPL at 0·5 per cent was mildly inhibitory while 0·25 per cent had no significant effect on the course of the fermentation. It is interesting to note that reducing sugar assays indicated no significant effect of BPL treatment on the initial reducing-sugar level.

A similar series of fermentation experiments was carried out with ethylene-oxide-treated medium. The cold Cryoxide was added to the unsterile flasks at the desired concentration and the flasks were shaken at 4°C for 2 h and then held in a 55°C water bath for

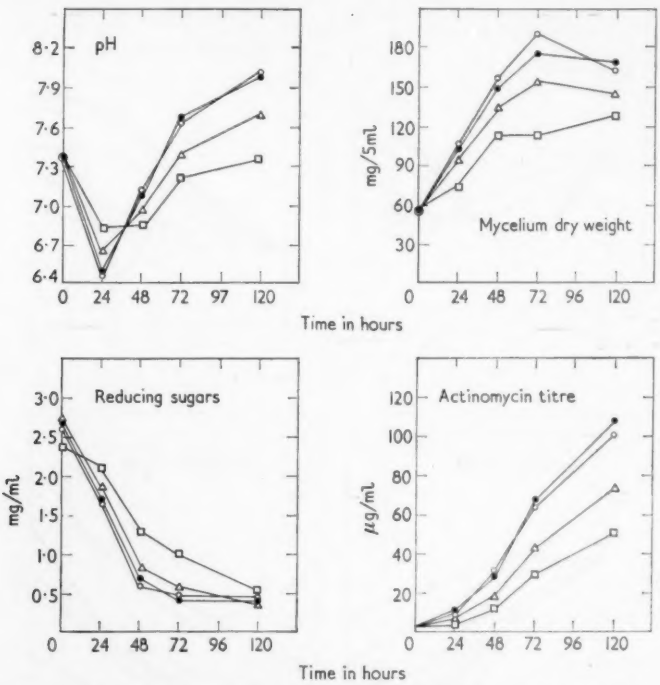


Fig. 2. Actinomycin fermentation with medium sterilized with beta-propiolactone. BPL %: ● none, ○ 0·25, △ 0·50, □ 1·0

1 h to vapourize the sterilant, followed by a 24-h incubation at 37°C; the unsterile flask surfaces and cotton plug were assumed to be sterilized during vapourization of the Cryoxide.

As with BPL, concentrations of 0·25–1 per cent ethylene oxide were generally effective in routine sterilization of the growth medium. The fermentation results were also similar to the BPL experiments in that 0·25 per cent ethylene oxide was non-toxic while higher doses resulted in reduced organism growth and lower antibiotic yields. However, in all comparative experiments the deleterious effects of ethylene oxide at the higher concentrations were greater than those observed for BPL. These results are illustrated in Figs. 3 and 4.

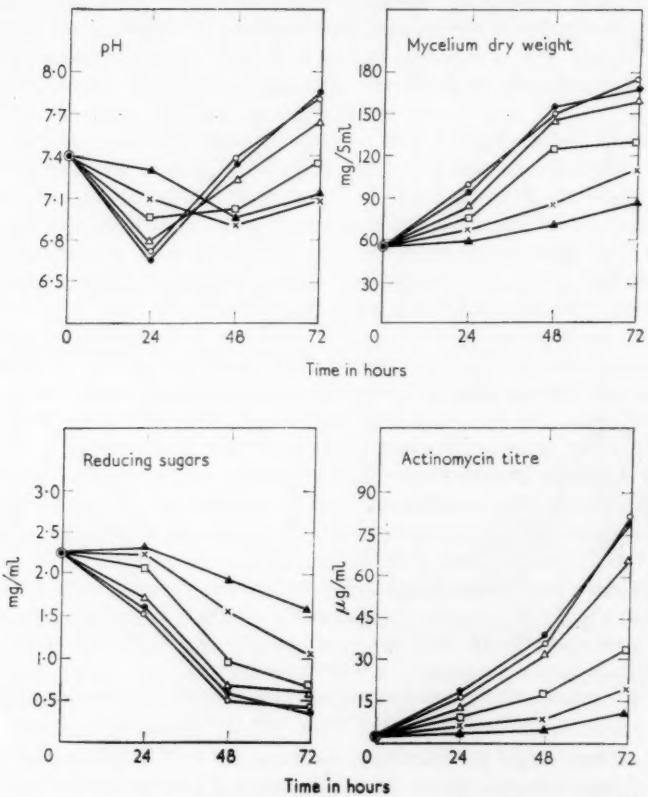


Fig. 3. Effect of ethylene oxide treatment on actinomycin fermentation. Medium autoclaved before treatment. Ethylene dioxide %: ● none, ○ 0.12, △ 0.25, □ 0.50, × 1.0, ▲ 2.0

Discussion

Both ethylene oxide and BPL have been shown to be strong biocidal compounds, effective against the resistant spores of *B. coagulans*. Both compounds can be used as routine sterilizing agents for fermentation media at concentrations of up to 0·5 per cent without significant effects on the growth-promoting qualities

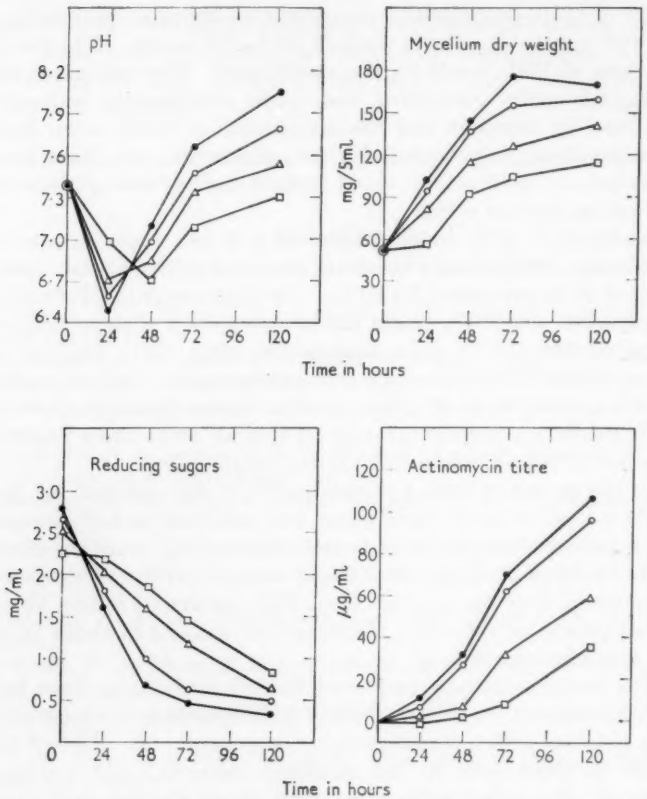


Fig. 4. Actinomycin fermentation with medium sterilized with ethylene oxide. Ethylene dioxide % : ● none, ○ 0.25, △ 0.5, □ 1.0

of the media. At concentrations required to sterilize large numbers of resistant spores (0·5–1 per cent), however, both agents have deleterious effects on fermentation. The experimental results also indicate that BPL is less damaging to the medium than ethylene oxide at these high biocidal concentrations.

Other advantages of BPL—the compound is a liquid that can be used at room temperature in contrast to the necessity of cooling the Cryoxide mixture and the medium to be treated to the boiling

point of 11°C—suggest this compound as the most promising of the two agents as a chem-sterilant for liquid media. The potential uses of BPL would be the sterilization of fermentation and biological media containing heat-labile components ordinarily sterilized by filtration and the sterilization of media when high-pressure steam is not available for autoclaving, or where high-temperature sterilization would impart undesirable qualities to the media, such as colour.

Assuming a BPL concentration of 0·25 per cent, the cost of sterilizing 1,000 gallons of medium would be $11.20 at the current price of $0.50 per pound for BPL. The time required for chemical sterilization with BPL would not be excessive if slightly elevated temperatures (50°C) were maintained after BPL addition to promote fast hydrolysis of the active compound. In these studies a 24-h incubation at 37°C was used to ensure hydrolysis. Since BPL exhibits a half-life of only 20 min at 50°C, the hydrolysis time could be reduced to 4–6 h at this temperature.

In the course of review of this paper, it was pointed out that while 0·5 per cent of either agent was sufficient to kill spores of *B. coagulans* in Eagle's medium no indication is given of the utility of this medium for subsequent tissue culture. Other investigators have found that the residues from BPL treatment inhibit subsequent growth of cells when these residues amount to about 10 mg per litre of medium.

The authors actually employed Eagle's medium in these tests only to examine the effectiveness of BPL against spores suspended in a medium with a serum protein component. No attempt was made to grow cells in the sterilized medium. Subsequently, however, the senior author has used serum treated with up to 0·15 per cent BPL in tissue culture media at the 10 per cent level without observing toxic effects. This is equivalent to 150 mg of BPL residue per litre of whole medium. These experiments were short-term (5–7 days) and it is not known whether repeated transfer of the cells in this medium would eventually produce toxicity.

The treated serum was added to the medium only after 24-h incubation at 37°C to ensure complete hydrolysis of the BPL. The effects of BPL added directly to the medium and placed in cell cultures without allowing for hydrolysis have also been tested. In this case, strong cytotoxicity is observed at 10–20 mg BPL per

litre. If care is taken to hydrolyze the BPL before testing, however, little or no cytotoxicity is observed at concentrations as high as 150 mg per litre. The cell lines used in these tests were the HeLa and Hep #2.

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Note

Inhibition of Microbiological Breakdown of $\Delta^{1,4}$-Androstadienedione with Cyclic Compounds

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According to the unanimous statement of several researchers, the $\Delta^{1,4}$-androstadienedione produced from various compounds by *Fusarium* strains undergoes oxidation to Δ^{1}-dehydrotestololactone.[1–6] As shown by our experiments, this later phase of

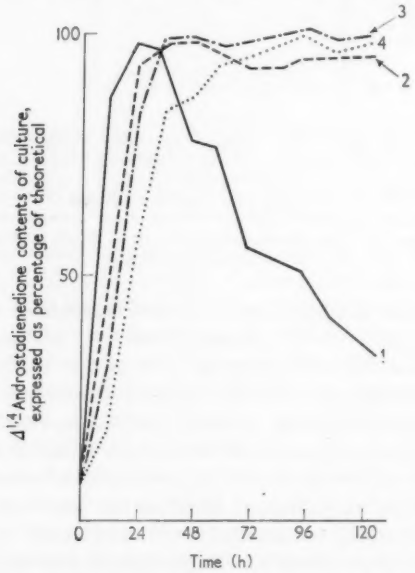

Fig. 1. Twenty-fold diluted cultures. Curve 1: 20 mg of Δ^4-androstenedione; curve 2: 20 mg of Δ^4-androstenedione + 0·5 mg of phenanthrene; curve 3: 20 mg of Δ^4-androstenedione + 2·0 mg of phenanthrene; curve 4: 20 mg of Δ^4-androstenedione + 5·0 mg of phenanthrene; each compound was dissolved in 1 ml of acetone

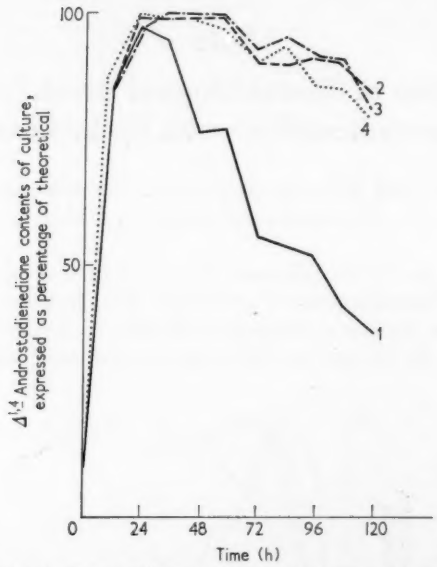

Fig. 2. Twenty-fold diluted cultures. Curve 1: 20 mg of Δ^4-androstenedione; curve 2: 20 mg of Δ^4-androstenedione + 0·5 mg of anthracene; curve 3: 20 mg of Δ^4-androstenedione + 2·0 mg of anthracene; curve 4: 20 mg of Δ^4-androstenedione + 5·0 mg of anthracene

oxidation can be inhibited by saturated steroids of the allo series and oestrone, but not by pregnandione.[5, 6] It has furthermore been found that this inhibitory action was due to allo compounds of varying structure.[5] On the basis of the above experimental data, we presumed that to produce inhibition, rings 'A' and 'B' had to be co-planar, while within certain limits the structure of the rest of the molecule was of no particular interest. Acting on this assumption, we examined whether the same effect could also be brought about by minor cyclic systems whose structure satisfied the above requirements. Experiments were carried out with *Fusarium caucasicum* strains. The methods of cultivation and analysis were identical to those described in earlier reports.[5, 6] Since the reported experiments had shown that inhibitory action was more effective in appropriately diluted cultures than in those

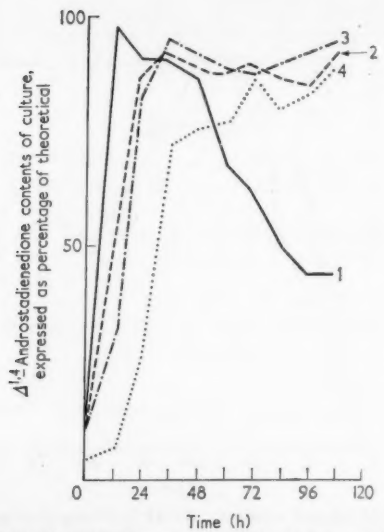

Fig. 3. Twenty-fold diluted cultures. Curve 1: 20 mg of Δ^4-androstenedione; curve 2: 20 mg of Δ^4-androstenedione + 0·5 mg of naphthalene; curve 3: 20 mg of Δ^4-androstenedione + 2·0 mg of naphthalene; curve 4: 20 mg of Δ^4-androstenedione + 5·0 mg of naphthalene

of the original concentration, in these studies 100 ml of culture diluted twenty-fold with distilled water in 500-ml conical flasks were used for each experiment. The oxygen supply to the cultures was ensured by a rotary shaker (200 rev/min) with a 2-cm stroke placed in a thermostat chamber at 28°C. The amounts employed and schedule are presented in the figures. We have tested the effects of phenanthrene, anthracene, naphthalene, beta-naphthol and phenol. The results are shown in Figs. 1 to 5.

Phenol was ineffective, but, as with the steroids, the co-planar minor cyclic systems were found to inhibit the production of Δ^1-dehydrotestololactone. Hence, to obtain the inhibitory effect a two-ring compound of appropriate structure is sufficient, and in the case of a three-ring compound it is of no importance whether the annelation of the three rings is linear or angular.

A detailed report on our experimental results is to appear in *Acta Microbiol. Acad. Sci. Hung.*

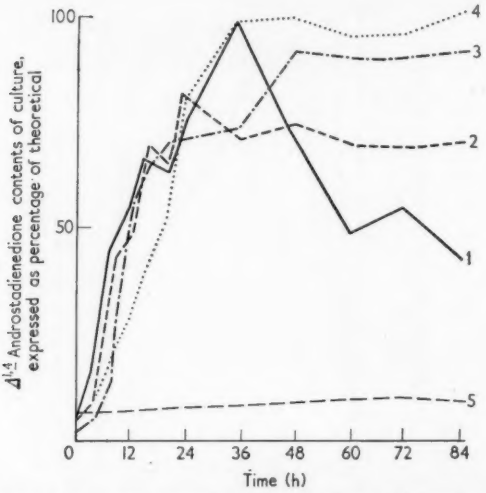

Fig. 4. Twenty-fold diluted cultures. Curve 1: 20 mg of progesterone; curve 2: 20 mg of progesterone + 0·5 mg of beta-naphthol; curve 3: 20 mg of progesterone + 2·0 mg beta-naphthol; curve 4: 20 mg of progesterone + 5·0 mg of beta-naphthol; curve 5: 5·0 mg of beta-naphthol

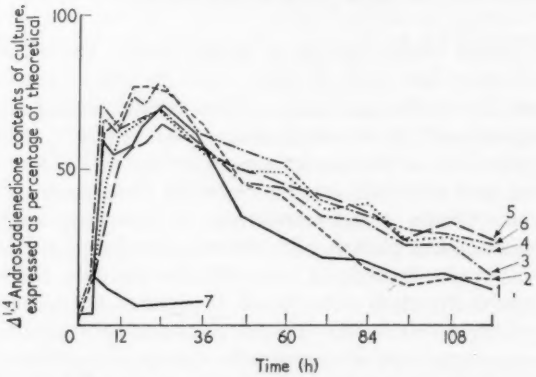

Fig. 5. Twenty-fold diluted cultures. Curve 1: 20 mg of progesterone; curve 2: 20 mg of progesterone + 0·5 mg of phenol; curve 3: 20 mg of progesterone + 2·0 mg of phenol; curve 4: 20 mg of progesterone + 5·0 mg of phenol; curve 5: 20 mg of progesterone + 10·0 mg of phenol; curve 6: 20 mg of progesterone + 20·0 mg of phenol; curve 7: 5.0 mg of phenol

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